VOLUME XX NOVEMBER 1959

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American Journal of

Veterinary RESEARCH

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In this issue . . .

- Low-Virulence Agents in Ornithosis Epornitics 935
- Acquired Resistance to Listeria Monocytogenes in Sheep 966
- Cobalt Pellet Therapy in Cattle 977
- Evaluation of Swine Erysipelas Bacterins and Vaccines 1002
- Nitrofurantoin for Canine Bacterial Nephritis 1020

CONTENTS ARE ON PAGES 929, 931, and 933

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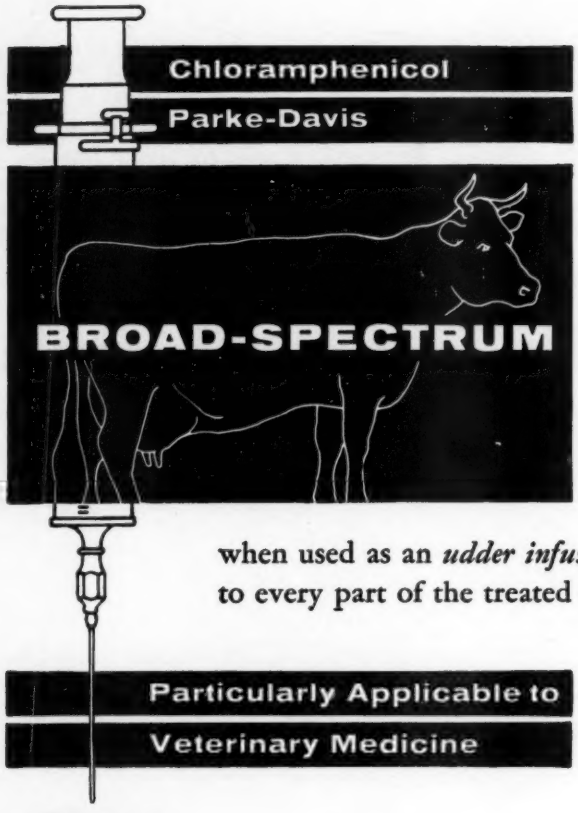

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Contents

The articles in the Research Journal are grouped, loosely, by animal species, followed by the more general articles. The articles listed on the cover are those that appear at the beginning of the various divisions or are particularly representative of a number of similar articles in a division.

Studies of Two Epornitics of Ornithosis Caused by Agents of Low Virulence..... 935
R. A. Bankowski and L. A. Page

Investigation of a Recent Ornithosis Epornitic in California Turkeys..... 941
L. A. Page and R. A. Bankowski

Studies on Pathogenic Newcastle Disease Virus Contaminants in Newcastle Disease Wing-Web Vaccines..... 946
A. S. Rosenwald, R. P. Hanson, and C. A. Brandly

A Study on the Oocystic Sporulation of Some Avian and Mammalian Eimeria Species Under Simulated Egyptian Summer Conditions, with Special Reference to the Cycle of Oocyst Production of *Eimeria Maxima*..... 954
M. H. Haiba and M. K. Selim

Studies of the Effect of Oxytetracycline on Chicken Spermatozoa..... 957
F. H. Wilcox

Spontaneous Cataracts in Turkeys..... 961
R. H. Rigdon, T. M. Ferguson, and J. R. Couch

Acquired Resistance of Sheep to *Listeria Monocytogenes* and Pilot Studies on Vaccination..... 966
J. W. Osebold, A. Njoku-Obi, and J. M. Abare

Antibiotics for the Control of Vibriosis of Ewes..... 973
F. W. Frank, W. A. Meinershagen, L. H. Scrivner, and J. W. Bailey

The Correction of Cobalt or Vitamin B_{12} Deficiency in Cattle by Cobalt Pellet Therapy..... 977
K. D. Skerman, A. K. Sutherland, M. W. O'Halloran, J. M. Bourke, and B. L. Munday

The Relationship of Nasal Discharge to Infection with Infectious Bovine Rhinotracheitis Virus..... 985
A. L. Brown and C. B. Bjornson

Serum Cholesterol and Paper Electrophoretic Lipoprotein Pattern in Cattle..... 989
K. Perk and K. Lobl

The Inactivation of Foot-and-Mouth Disease Virus, Type A-119, with *Beta*-Propiolactone..... 992
O. N. Fellowes, A. G. Edward, J. Tessler, G. C. Poppensiek, and J. B. Sharp

(Contents continued on page 931)

XUM

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XUM

(Contents—Continued from page 929)

An Evaluation of the Complement-Fixation Test in the Study of Foot-and-Mouth Disease 996
M. Savan

Comparative Experimental Evaluation of Swine Erysipelas Bacterins and Vaccines in Weanling Pigs, with Particular Reference to the Status of Their Dams 1002
R. D. Shuman

Action of Certain Viruses, *Mycoplasma Hyorhinis*, and Nasal Trichomonads on Swine Tissue Cultures 1010
W. P. Switzer

Effect of Nitrofurantoin on Experimental Canine Bacterial Nephritis 1020
E. H. Coles and J. E. Mosier

An Epidemiological Study of Canine Lymphoma and Its Public Health Significance 1026
D. Cohen, S. Booth, and O. Sussman

The Oral and Pharyngeal Pathology of Domestic Animals—A Study of 487 Cases 1032
R. J. Gorlin, C. N. Barron, A. P. Chaudhry, and J. L. Clark

The Absorption of Red Blood Cells After Parenteral Injection at Various Sites 1062
C. H. Clark and C. H. Woodley

A Comparison of Blood Volumes as Measured by Rose Bengal, T-1824 (Evans Blue), Radiochromium-Tagged Erythrocytes, and a Combination of the Latter Two 1067
C. H. Clark and C. H. Woodley

The Effects of Certain Diseases and Transfusion Methods on the Life Span of Red Blood Cells 1069
C. H. Clark and C. H. Woodley

(Continued on page 933)

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The Council on Research of the AVMA announces the availability of a number of fellowships for postgraduate training for the academic year, 1960–1961.

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(Contents—Continued from page 931)

Nephelometry and a Nephelo-Culture Flask Used in Measuring Growth of Leptospires 1072
H. C. Ellinghausen, Jr.

The Identification of Viable and Nonviable *Erysipelothrix Insidiosa* with Fluorescent Antibody 1077
J. D. Marshall, W. C. Eveland, and C. W. Smith

Hematological Effects of Acute and Chronic Experimental Blood Loss in the *Macaca Mulatta* Monkey 1081
G. M. Krise and N. Wald

The Digestibility of Animal Fats and Proteins by Mink 1086
W. L. Leoschke

Scientific Notes 1090

Serologic Classification of *Escherichia Coli*—*P. J. Glantz* 1090

Flocculation Tests for Pullorum Disease. II. A Study of the Stability of Frozen Antigen Extract for Conducting the Microscopic Slide Test— 1090
E. P. Roznowski and V. D. Foltz

Research Tools 1091

Index 1095

Classified Advertisements

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Studies of Two Epornitics of Ornithosis Caused by Agents of Low Virulence

R. A. BANKOWSKI, D.V.M., Ph.D., and L. A. PAGE, Ph.D.

Davis, California

ORNITHOSIS AGENTS recovered from turkey flocks in at least three states were characterized by a high pathogenicity for turkeys and laboratory animals and an apparently high infectivity for handlers of diseased carcasses.[3,5,7,8,9] This report describes the first two proven epornitics* in California turkey flocks in which the ornithosis agents isolated were characterized by a relatively low pathogenicity for turkeys and laboratory animals. In each instance, the disease in the flock was mild—slight depression and loss of a few birds (less than 1%)—yet the poultryman submitted a few birds to a diagnostic laboratory for examination. There were no known infections among persons handling infected birds on the premises or during processing, unlike the mild epornitics in turkeys in Wisconsin recently reported by Grabar.[4] The mildness of the disease in California turkeys, in contrast to the disease reported elsewhere, merited this study.

HISTORY AND DIAGNOSIS

Flock A.—The flock consisted of 10,000 Broad-Breasted Bronze turkeys, of which 4,000 were used as breeding hens. Over 1,900 turkeys were processed in the fall of 1954, when the birds were about 6 months old. The inspecting veterinarian noticed an unusual type of aerosacculitis and asked the veterinarians of the State Diagnostic Laboratory to assist him in interpretation. A large number of birds had slightly enlarged, clay-colored livers and purple discoloration of the spleens. No aerobic bacteria were isolated from these organs. Ornithosis was suspected because of some pericarditis and fibrinous plaques on the hearts and livers. Observations of the remaining flock did not reveal signs of major illness; however, the owner reported a slight shrinkage of the breast muscles and a 10 per cent drop in feed consumption in the birds a few days before processing them.

While diagnosis was pending, two groups of 1,200 birds each were marketed on Dec. 3 and 9, 1954. Similar liver and heart lesions were observed, and three complete carcasses and about 1,000 lb. of livers and hearts were condemned.

Affected organs were submitted to the G. W. Hooper Foundation, San Francisco, Calif., for examination. Lesions revealed intracellular elementary bodies in smears stained by Macchiavello's method, and a presumptive diagnosis of ornithosis was made. Inoculation of organ suspensions and exudates produced ornithosis lesions in 1 of 5 mice. Spleens and livers of the mice with the first passage were readily infectious and pathogenic for mice in subsequent passages by the intraperitoneal route. The isolate was designated as C-1 turkey ornithosis agent.

On December 10, a broad-spectrum antibiotic was incorporated into the diet at the rate of 300 Gm. per ton, and on December 27, 17 days after treatment was initiated, a fourth group of turkeys was submitted for processing. Of these, about 80 per cent showed typical fibrinous exudates on the heart or liver or both; plaques showed evidence of degeneration. A section of a heart plaque showed elementary bodies within the exudate (fig. 1).

In February, 1955, two months after the diagnosis, blood samples for serologic studies were taken from 115 birds, representing a cross section of the remaining flock. Nine of the 115 serums gave low positive reactions by the indirect complement-fixation (ICF) test,[6] with titers ranging from 1:2 to 1:16. The remaining serums were negative. The 9 reactors and 6 other birds from the flock were brought to the laboratory and held

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This investigation was supported in part by a grant from the Agricultural Research Service, U.S.D.A., Washington, D.C.
* An epizootic specifically affecting fowls.

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untreated as a group in isolation for further studies.

Flock B.—The owner had maintained a breeding flock of Broad-Breasted Bronze turkeys for 14 years without any significant disease problem. On Jan. 29, 1956, 4 birds in a flock of 1,000 breeders were found dead, and 20 birds were described as listless. Over-all egg production and feed intake were unaffected. The dead birds were sent to the State Poultry Diagnostic Laboratory at Sacramento, Calif., for examination. The pathologist reported a preliminary diagnosis of "pneumonia of undetermined cause." He noticed a severe bilateral red consolidation of the lungs, a copious caseofibrinous exudate in the pericardial sac and anterior air sacs, moderate hepatomegaly, and splenomegaly. Smears of the exudate did not reveal elementary bodies.

During the next 12 days, 7 more birds with similar gross lesions died; this time the pathologist reported that smears of exudates made from the tissues contained elementary bodies characteristic of ornithosis. Three more birds died, bringing the total mortality to 14. Sporadic coughing was noticed in sick birds, but egg production and feed intake were still unaffected. On February 16, serum samples from 6 birds were submitted to the diagnostic laboratory of the School of Veterinary Medicine, University of California, Davies, for serologic testing. The ICF titers ranged from 1:2 to 1:512. On February 25, the birds were placed for 30 days on a diet containing 400 Gm. of chlortetracycline (Aureomycin *) per ton.

Twenty-nine days after the initial mortality and prior to treatment, 3 live birds were obtained from the flock for postmortem examination. All were well-fleshed. The ICF titers for ornithosis of these serums were 1:256 or greater, 1:256, and 1:16, respectively.

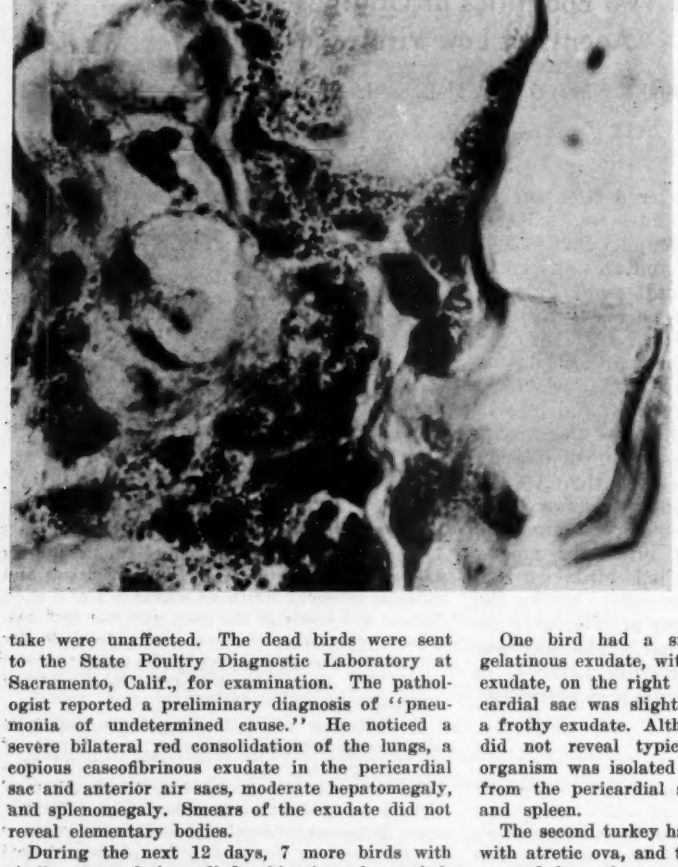

Fig. 1—Section of a plaque on a turkey heart, illustrating the intracellular elementary bodies in the exudate.

One bird had a small amount of yellowish, gelatinous exudate, with small granules of caseous exudate, on the right thoracic air sac. The pericardial sac was slightly thickened and contained a frothy exudate. Although smears of the exudate did not reveal typical elementary bodies, the organism was isolated from suspensions prepared from the pericardial sac, kidney, air sacs, liver, and spleen.

The second turkey had a recent acute peritonitis with atretic ova, and the ornithosis agent was recovered from the mesentery, cloacal contents, and the ova. No lesions were visible in a third bird, although the ornithosis agent was readily recovered from most of the tissues. The isolate was designated as C-2 turkey ornithosis agent.

Cultures of the organs and exudates of all 3 birds revealed no pleuropneumonia-like organisms (PPLO), and the serums were negative to the agglutination test.

Pigeons roosting in a barn near the turkey quarters were known to have intermingled with

* Produced by American Cyanamid Co., New York, N.Y.

the turkey flock. Fifteen pigeons and 2 squabs were trapped on March 3. None of the birds had gross lesions. Serums of 5 adult pigeons presented CF titers of 0 to 1:16, and the remaining serums showed titers of 1:64 to 1:256. The ornithosis agent was isolated by mouse passage tests from the organs of 3 of the pigeons. This isolate was designated CP-2 pigeon ornithosis agent.

For further studies to determine whether progeny of the known infected breeding flock carried ornithosis antibodies or agents, 100 poults hatched from eggs collected just before chlortetracycline was fed to the infected flock were placed in laboratory isolation when 1 day old.

RESULTS

FLOCK A

Persistence of Serologic Activity.—Ornithosis antibodies were detected for at least 15 months, but not more than 25½ months, in some of the serums of a group of 9 turkeys that had reacted to the ICF test two months following diagnosis of the disease in the flock (table 1).

To determine whether titers might change in the presence of another infection, 9 turkeys were inoculated with a virulent culture of avian Mycoplasma (PPLO). A severe sinusitis developed in all recipients by the fourteenth day; however, no significant changes in ICF titers were noticed in tests made 25 days after inoculation. A progressive decrease in titer was noticed in the PPLO-inoculated and uninoculated turkeys.

During the experiment, 17 months after initial diagnosis, the turkeys were skin-tested with Benedict's [8] detergent-extracted psittacosis antigen (table 1). Twelve of the 13 tested birds exhibited a dermal sensitivity to this antigen. Two uninfected control birds (not tabulated) were completely negative to the test.

Although the ICF antibody titers persisted for a considerable time following the natural epornitic, the antibodies were not detected by the ICF test after challenge with homologous organisms.

The Persistence of Immunity to Challenge with the Homologous Organism.—To test the immunity of recovered turkeys to ornithosis reinfection, the 9 turkeys retained for the experiment were subjected to an intratracheal challenge with a suspension of the homologous organism. The inoculum contained 44,300 mouse i.d.$_{50}$. Included in the test was a second group of 9 turkeys, 24 months old, that had been experimentally infected with the virulent NJ-1 agent when 3 months old and a third group of 9 turkeys, 13 months old, with no previous history of ornithosis (table 2).

During the 53-day observation period, all three groups appeared normal. Rectal temperatures, taken every other day, showed a few turkeys in each group with a fever during the second and third week. Turkeys in groups 2 and 3 showed ICF titers that developed in the second to third week; however, those in group 1 (homologous organism) showed no ICF antibodies throughout the 53-day period. To determine the extent of the disease and the

TABLE 1—Persistence of Indirect Complement-Fixation (ICF) Titers and Skin Test Reactions in Turkeys Tested Over a Period of 28.5 Months Following Natural Infection with C-1 Ornithosis Agent

Turkey No.	Reciprocal ICF titer and treatm n									Skin test
	2/10/55*	2/28/55†	3/1/55	3/25/55**	5/1/56	3/28/57	5/1/57	5/3/57	6/25/57	5/12/56
1	4	8	0	0	0	0	0	All	0	+
2	16	64	Infectious	8	..	0	0	challenged	0	++
3	16	64	sinusitis	4	4	0	0	with 44,300	0	+
4	8	8	induced	4	2	D	..	mouse i.d.$_{50}$	0	++
5	8	16	experi-	4	..	..	..	homologous	..	...
6	0	2	mentally	0	0	D	..	ornithosis	..	±
7	0	32		4	2	0	0	agent	0	+
8	0	0		0	..	D	..		..	±
9	0	0		0	..	..	..		..	...
10	0	0	Uninocu-	0	0	D	..		..	±
11	2	0	lated	0	0	0	0		0	++
12	0	0	controls	0	0	0	0		0	++
13	2	0		0	0	0	0		0	Neg.
14	4	4		0	0	0	0		0	++
15	4	0		0	0	0	0		0	++

* Diet contained 300 Gm. of broad-spectrum antibiotics per ton of feed for previous two months. † Antibiotic-free diet given since last blood sample (Feb. 10, 1955). ** Severe sinusitis in all inoculated birds by the fourteenth day.
+ = erythema with minimal edematous reaction; ++ = erythema, 25 mm. or more in diameter, with marked edema; ± = slightly edematous tissue beyond inoculated area; D = destroyed.

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presence of ornithosis agents, 3 turkeys from each group were killed on the fifth, fourteenth, and 53rd days following inoculation with the challenge organism. None had severe lesions of ornithosis. Lesions were slightly more extensive in turkeys of groups 2 and 3 than in group 1; however, the lesions found in birds in groups 2 and 3 resembled those frequently associated with ornithosis. Fourteen days after challange inoculations, the agent was recovered from affected tissues and organs from 1 turkey in group 2 and 3 turkeys in group 3. No ornithosis agents were isolated from birds in group 1.

The results of the cross-immunity study were not decisive but did indicate the possibility of immunogenic differences between the two strains.

To establish possible immunogenic differences, a cross-immunization experiment was performed in mice with the C-1 and NJ-1 ornithosis agents (table 3). Sixty mice were inoculated with each strain of the agent, which consisted of a suspension of formalin-killed organisms propagated in mouse tissue. Two intraperitoneal injections were given nine days apart and a final dose of live agent was injected intraperitoneally on day 18. Although no signs were observed after inoculation with the live C-1 strain of the agent, 31 mice died after inoculation with the NJ-1 strain. The mice that survived were divided in groups and challenged 46 days after the last dose. As summarized (table 3), mice inoculated with the more virulent NJ-1 strain were immune to challenge with the homologous agent, but were completely susceptible to the mild C-1 strain. In contrast, mice given the C-1 strain were protected against the homologue, but over one half (11 of 20) were still susceptible to the NJ-1 strain. These results did not give undisputed evidence of an immunologic distinctness between the two strains, since the C-1 strain persisted longer than the highly pathogenic NJ-1 strain in the unchallenged control groups. The experiment, however, lends support to the conclusion that these orni-

TABLE 2—Results of Intratracheal Challenge of Ornithosis-Recovered Turkeys with C-1 Ornithosis Agent

History	Turkey No.	Reciprocal ICF titer: Days postchallenge						Postmortem results		
		−2†	5	14	24	46	53	Day killed	Ornithosis lesions	Agent isolation
GROUP 1										
3-year-old hens naturally infected with C-1 agent when 6 months old.	14	0	0	....	....	....	....	5	0	0
	3	0	0	....	....	....	....	5	0	0
	1	0	0	....	....	....	....	5	0	0
	15	0	....	0	....	....	....	14	±	0
	2	0	....	0	....	....	....	14	±	0
	7	0	....	0	....	....	....	14	±	0
	13	0	....	....	0	0	0	53	±	0
	12	0	....	....	0	0	....	D50	....	0
	11	0	....	....	0	0	0	53	+	0
GROUP 2										
2 toms, 7 hens, 24 months old. Experimentally infected at 3 months of age with NJ-1 ornithosis agent.	1	0	0	....	....	....	....	5	+	0
	2	0	0	....	....	....	....	5	+	0
	3	0	0	....	....	....	....	5	+	0
	4	0	....	0	....	....	....	14	0	0
	5	0	....	128+	....	....	....	14	++	+
	6	0	....	0	....	....	....	14	±	0
	7	0	....	....	0	0	0	53	++	0
	8	0	....	....	2++	0	8	53	±	0
	9	0	....	....	128+	128	64	53	0	0
GROUP 3 (CONTROLS)										
9 hens, 13 months old, no previous history of ornithosis.	1	0	0**	....	....	....	....	5	±	0
	2	0	0**	....	....	....	....	5	0	0
	3	0	0**	....	....	....	....	5	0	0
	4	0**	....	....	....	....	....	D9	++	0
	5	0	....	0**	....	....	....	14	±	+
	6	0	....	0**	....	....	....	14	0	+
	7	0	....	0	....	....	....	14	0	+
	8	0	....	....	64	512	128	53	±	0
	9	0	....	....	128	512	256	53	++	0

* Turkey numbers in group 1 correspond to numbers in table 1. † Two days before challenge. ** Serum anticomplementary.

± = slightly cloudy or thickened air sacs; + = fibrinous mycardial plaques typical of ornithosis or ruptured ova with evidence of mild inflammatory reaction in peritoneum; ++ = acute or resorbing fibrinous plaques on heart or liver with or without adhesions (air sacs may or may not show mild inflammatory reaction); D = died; ICF = indirect complement-fixation.

thosis agents have detectable immunologic differences.

Flock B

In the second epornitic, which involved an agent of low virulence, more information was needed to determine the possibility of transovarian transmission.

One hundred 1-day-old poults, hatched from eggs collected after isolation of the ornithosis agent but before the birds were fed chlortetracycline, were placed in a clean isolated area. Over a 321-day period, blood samples were taken at intervals for serologic studies and attempts were made to isolate the ornithosis agent. No antibodies or disease agents were demonstrated in the birds. Twenty-five poults, when 82 days old, were subjected to artificially produced cold drafts and wetness for four to eight hours daily in an attempt to subject them to stress. Eleven days later the birds were killed. No lesions were observed, no ornithosis agent was isolated, and no birds developed any serologic response. Another group of birds was allowed to mature, but the stress of egg production or natural physiologic maturation failed to precipitate any potential latent ornithosis.

TABLE 3—Cross-Immunization Study in Mice with NJ-1 and C-1 Agents Causing Ornithosis in Turkeys

Immunization strain	No. mice surviving immunization	Challenge strain	Results of challenge: No. deaths	No. with pathologic residue	Virus isolated*
NJ-1	29/60	NJ-1†	0/10	0/10	0
		C-1**	0/10	10/10	+
		None	0/9	0/9	0
C-1	60/60	C-1	0/20	2/20	0
		NJ-1	0/20	11/20	+
		None	0/20	3/20	+

* Pool of spleens 14 days after challenge. † 4,300 mouse l.d.$_{50}$. ** 1,500 mouse i.d.$_{50}$.

Discussion

The two epornitics of ornithosis in California turkey flocks were diagnosed by accident, at a time when diagnostic and research facilities for this disease were inadequate. The mildness of the disease in contrast to the disease in turkeys in other states emphasized the need for further research on ornithosis caused by agents of low virulence. From the results reported here, it was apparent that, in spite of heavy medication, approximately 8 per cent of a representative sample from the naturally infected flock presented low ICF titers (highest, 1:32) 63 days after the disease was diagnosed. Serum antibodies persisted within this small group for at least 17 months. The persistence of ICF antibodies was in contrast to data reported by Page,[4] who found that ICF antibodies persisted for at least five but not more than 15 months following experimental infection with the more virulent NJ-1 strain of the ornithosis agent. It was significant that some of the ICF reactor turkeys showed an increase in titer when removed from medicated mash, although titers were unaffected following an experimentally induced severe sinusitis infection.

TABLE 4—Presence of Ornithosis Organisms or Antibodies in Progeny of Ornithosis-Infected Turkeys

Age of birds (days)	Procedure	Ornithosis agent isolation results	ICF* results
1	25 poults killed. Tissues removed for agent isolation studies.	Negative	.....
47	25 poults killed. Tissues removed for agent isolation studies. Blood collected for serologic testing.	Negative	Negative
2–80	9 poults died of causes unrelated to experiment. Tissues removed for agent isolation and blood for serologic testing.	Negative	Negative
82	25 poults isolated and subjected to artificially produced cold drafts and wetness four to eight hours daily for seven days. Injected with cortisone, 40 mg. per bird. Eleven days after cold-wet treatment, birds were killed. Tissues removed for agent isolation and blood collected for serologic testing.	Negative	Negative
231	8 males killed. Tissues removed for agent isolation and blood for serologic testing.	Negative	Negative
321	5 males and 1 female killed. Tissues removed for agent isolation and blood for serologic testing.	Negative	Negative

* ICF = Indirect complement-fixation test.

The fact that some immunogenic differences were observed between the NJ-1 and the California C-1 strain in the cross-immunity studies in turkeys and mice revealed a need for investigating the characteristics of agents that may be encountered in turkeys under field conditions.

Davis and Delaplane [9] were unable to demonstrate transovarian passage of ornithosis in breeder turkeys experimentally infected with a highly pathogenic strain. Since most ornithosis agents are known to be lethal to developing embryos, it was conceivable that an agent of relatively low virulence would be more likely to be egg-transmitted. Ornithosis antibodies or organisms could not be demonstrated in 100 poults hatched from eggs collected during the height of the disease in spite of exposure of some of the progeny to adverse conditions.

SUMMARY

Investigation of two epornitics of ornithosis in turkeys caused by agents of low virulence revealed the following:

1) Indirect complement-fixation (ICF) titers were found in approximately 8 per cent of a sample of breeders tested two months after diagnosis. Antibodies persisted as long as 17 months after the natural infection, and a cutaneous hypersensitivity to Benedict's ornithosis skin-test reagent was present for at least 18 months.

2) Experimentally induced infectious sinusitis in convalescent turkeys, six to eight months after diagnosis of ornithosis infection, did not alter ICF titers of the birds and did not appear to reactivate any potential latent disease.

3) Turkeys resisted reinfection and antigenic stimulation following 44,300 mouse $l.d._{50}$ of the homologous strain given intratracheally 30 months after natural exposure. However, multiplication of the agent and a serologic response were evident in mature, apparently normal controls and in turkeys previously exposed to the more virulent NJ-1 ornithosis agent.

4) All attempts to isolate an infectious agent or demonstrate positive ornithosis serology in 100 poults hatched from eggs collected during the peak of an ornithosis epornitic in the parent flock were unsuccessful.

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Investigation of a Recent Ornithosis Epornitic in California Turkeys

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ORNITHOSIS has been diagnosed in turkeys in California every two years since 1954. The agents responsible have been of low virulence and have not caused detectable human disease or serious economic loss. This is in contrast to outbreaks in Texas, New Jersey, and Oregon.[1,2,7] The most recent epornitic* in turkeys in California was on a ranch in the central part of the state in February, 1958. This report describes the epornitic, studies on the pathogenicity of the disease agent for various laboratory animals, and attempts to isolate an egg-transmitted organism.

HISTORY

Clinical Aspects.—The flock consisted of about 400 toms and 1,700 breeder hens, which had been in production for three months. The birds' condition was characterized by a listless appearance, yellow-green droppings, and a tendency to gasp when handled. Egg production dropped 50 per cent, though feed consumption remained normal. Mortality increased sharply after clinical signs appeared. A total of 58 birds died during the course of the disease. Mortality continued in spite of chlortetracycline given in feed and water (500 Gm./ton of feed; 0.1 Gm./gal. of water) for four days prior to diagnosis, although it temporarily improved the appearance of the birds.

Diagnosis.—Serum samples from the flock were sent to the diagnostic laboratory of the School of Veterinary Medicine, University of California, Davis, for serologic tests for ornithosis. Serologic tests for chronic respiratory disease and Newcastle disease proved negative. The indirect complement-fixation (ICF) test* on 75 serum samples indicated an over-all reactor rate of 93.4 per cent, with 52.0 per cent showing titers ranging from 1:64 to 1:2,048. A tentative diagnosis of ornithosis was made.

Four affected hen turkeys were obtained from the infected flock and necropsied. Each had ruptured yolk sacs; 3 had aerosacculitis. The birds most severely affected had fibrinous plaques on the heart and liver, hepatomegaly, and diffuse, foamy aerosacculitis and tracheitis. The spleen was enlarged, hemorrhagic, and mottled. The kidneys were swollen and red, and the ureters contained an excess of urates (fig. 1, 2).

The ICF titers of these 4 turkeys varied from 1:16 to 1:128. Pericardial sacs and kidneys of each bird were triturated in a mortar with an aluminum oxide (Alundum*) and beef heart broth. The tissue suspensions were inoculated into 16- to 20-Gm. Webster mice for virus isolation. Within seven days, lesions characteristic of ornithosis were seen in six of eight sets of inoculated mice. Bacteria-free smears of the mouse exudates, stained by Macchiavello's or Castañeda's methods, revealed intracellular elementary bodies, confirming the tentative diagnosis of ornithosis. The new isolate was labeled California-4 (C-4) turkey ornithosis agent.

Treatment and Disposal of the Flock.—The flock was treated with chlortetracycline (400 Gm./ton of feed) for five days early in March, 1958, and then marketed. When the hens were processed, the carcasses were of high quality, although healed lesions were observed on some of the hearts. Extensive fibrinous epicarditis was found in a majority of the toms, and the organs of more than half were condemned. Attempts to isolate the organism were negative on random samples of hearts and kidneys from the hens and lesion-bearing hearts from the toms.

RESULTS

Characterization and Pathogenicity of the C-4 Agent for Various Species of Animals.—The agent causing ornithosis in turkeys was classified as a member of the psittacosis-lymphogranuloma group *(Miyagawanella ornithosis)* on the following bases:

1) The disease agent was originally isolated from the pericardial sac, kidney, and spleen of a serologically positive turkey showing pericarditis, perihepatitis, and bacteria-free pericardial and peritoneal exudates containing intracellular elementary bodies.

2) The disease agent was an intracellular parasite positive to Castañeda's and Macchiavello's stains, and was morpho-

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The assistance of Dr. H. E. Adler, to whom the case was originally submitted, is gratefully acknowledged.

* An epizootic specifically affecting fowls.

* Alundum is produced by the Norton Co., Worcester, Mass.

Fig. 1—Gross lesions of a turkey naturally infected with the C-4 turkey ornithosis agent. Arrows indicate fibrinous plaques on the heart and liver.

logically similar to organisms of the genus Miyagawanella.

3) Intraperitoneal inoculation of the disease agent into mice resulted in characteristic lesions of psittacosis as described by Meyer.[4]

4) Intraperitoneal inoculation of the isolated disease agent in sufficient numbers into turkeys caused a systemic disease characterized by pericarditis, perihepatitis, and aerosacculitis. Triturated turkey tissues, suspended in broth, were inoculated intraperitoneally into mice. Typical lesions of ornithosis were observed in the mice, and the disease agent was recovered.

5) In turkeys and pigeons, the disease agent stimulated the production of antibodies which were detected by serologic tests using known ornithosis and psittacosis microorganisms as antigens.

6) The isolated disease agent was lethal to chicken embryos when inoculated by the yolk-sac route.

7) Parakeets given the disease agent intraperitoneally developed typical lesions of acute psittacosis. The agent was readily isolated from mice which were given infective parakeet tissues.

The pathogenicity of the C-4 ornithosis agent for laboratory animals was determined by inoculating a suspension of the disease agent into two species of birds, three species of mammals, and chicken embryos (table 1). The suspension, prepared from infected spleens and livers of mice, was decimally diluted and inoculated in 0.1-ml. amounts into embryonating chicken eggs, mice, guinea pigs, poults, pigeons, and parakeets. The suspension

Fig. 2—Enlarged, mottled spleen and foamy, thickened, abdominal air sacs of the turkey shown in figure 1.

was not titrated in the last species. The agent produced a fatal infection when inoculated intracerebrally into pigeons, intraperitoneally into parakeets, and via the yolk sac into chicken embryos. When the agent was inoculated by the intraperitoneal route into mice or turkeys, few clinical signs resulted, though gross lesions were observed at necropsy.

Egg Transmission Studies.—To study the possibility of transovarian passage of ornithosis in turkeys, 535 eggs were collected during the acute phase of the ornithosis epornitic. The eggs were incubated and candled daily after the seventh day. Forty-one per cent of the eggs were infertile. A sampling of live embryos, removed at weekly intervals, was used in attempts to isolate the disease agent by mouse-passage tests. Isolation attempts were also made on the organs or yolk sacs of all dead embryos and on 13 poults as they hatched (table 2). Blood serum was collected for serologic tests from all live embryos removed at the third week of incubation, as well as from day-old poults. Eight poults hatched from the eggs were maintained for further serologic tests.

The ornithosis agent could not be isolated from either 262 embryo tissues examined at various ages or from 13-day-old poults. The blood serum of 2 embryos, 23 days old, had an ICF titer of 1:4. Antibody was also detected in the amniotic fluid, but not in the chorioallantoic fluid of 1 of these serologically positive embryos. High ICF titers were detected in the serums of 4 poults, 1 day old; 1 had a titer greater than 1:512, and 3 others exhibited partial titers to 1:64.

Ecology.—Since ornithosis appeared suddenly in the turkeys on this ranch, and the source of infection was not readily apparent, a search for a carrier host was made. The grower could not recall any unusual influx of migratory birds. Blackbirds were seen occasionally on the ranch, and a small flock of sparrows was known to nest in a tree near the turkey pens. A few birds of each species were shot one

TABLE 1—Six Species of Animals Given 0.1-ml. Inoculations of a Suspension of C-4 Turkey Ornithosis Agent in Varying Dilutions

		Species							
		Chicken embryos (7-day)	Swiss albino mice (4 weeks old)	Guinea pigs (250 Gm.)	White cross poults (12 weeks old)	White King pigeons (12 months old)		Adult parakeets	
Dilution of suspension	No. mouse $i.d._{50}$ injected	death/ inoc.	lesions/ inoc.	lesions/ inoc.	lesions/ inoc.	death/ inoc.	lesions/ inoc.	death/ inoc.	lesions/ inoc.
10^{-1}	6,800	.....	5/5	0/5	5/5	5/5	5/5	1/4	4/4
10^{-2}	680	4/5	5/5	0/5	.....	.....	.....	.....	.....
10^{-3}	68	3/4	5/5	0/5	0/5	4/5	5/5	.....	.....
10^{-4}	6	3/5	5/5	0/5	.....	.....	.....	.....	.....
10^{-5}	Less than 1	2/5	2/5	0/5	0/5	2/5	4/5	.....	.....
$l.d._{50}$ or $i.d._{50}$ per 0.1 ml.	.....	10,000	68,000	.....	100	23,000	>100,000	.....	.....
Pathology	.....	Hemorrhagic embryos	Enlarged spleen, liver; fibrinous ascites	None	Severe pericarditis and perihepatitis in 10^{-1} birds	Severe inflammation —brain surface		Severe fibrinous perihepatitis, aerosacculitis, peritonitis; splenomegaly	
Clinical signs	.....	.....	Ruffled fur; enlarged abdomen	None	None	Bilious green diarrhea; ataxia, depression, anorexia		Depressed, off-feed	
Serologic response at two weeks	.....	.....	.....	CF Negative	4/5 agglutination positive in 10^{-1} group	2/5 CF positive in surviving birds of 10^{-3} group		.....	
Ornithosis agent recovery	.....	+	+	.....	+	+		+	

Inoculations were made by the intraperitoneal route except in the pigeons, which were inoculated intracerebrally, and the chicken embryos, which were inoculated via the yolk sac.

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month after the turkey epornitic was diagnosed.* Examination of their organs by mouse-passage tests revealed that the sparrows, although lesionless, harbored a disease agent causing typical lesions of ornithosis in mice. The characterization of this agent will be reported later.

DISCUSSION

The virulence of an ornithosis agent in a turkey epornitic appeared to be directly related to its pathogenicity for certain laboratory animals. An ornithosis agent isolated from a turkey in Texas, where mortality rates in affected flocks exceeded 7 per cent,[7] regularly produced fatal infections in guinea pigs, mice, and rice birds after intraperitoneal inoculation but did not affect pigeons by the intracerebral route.[9] The high degree of pathogenicity of this ornithosis agent for laboratory animals was attributed to its toxin-producing ability, as measured by intravenous mouse inoculation. Other strains of avian or mammalian origin, except those isolated from egrets, demonstrated relatively less toxigenicity.

Another toxigenic strain, NJ-1, was shown to be highly pathogenic for turkeys; the relative pathogenicity of this New Jersey strain for mice was compared with the C-1, C-2, and CP-2 (pigeon) California strains.[10,11] All of these studies point to a relationship between the toxigenicity of the microorganism and its pathogenicity for various species.

Although the ability of the C-4 turkey ornithosis agent to produce toxin was not measured, this organism appeared to have a relatively low virulence when compared to ornithosis organisms isolated from turkeys in Texas and New Jersey. Mortality in the flock originally affected with this disease agent was less than 3 per cent. In contrast to the Texas or New Jersey turkey isolates, the C-4 agent was infectious but not lethal to either mice or turkeys after intraperitoneal inoculation of over 6,000 mouse $i.d._{50}$, and was innocuous to guinea pigs by the same route. Small numbers of the C-4 agent produced fatal infections in pigeons by the intracerebral route and were irregularly fatal to developing chicken embryos.

* Courtesy of W. F. Rooney, extension poultry specialist, University of California, Davis.

This report adds to present evidence that transovarian passage of ornithosis does not occur or is extremely rare. Studies by Davis and Delaplane[4,5] indicate that ornithosis agents were not recovered from eggs collected from hens artificially infected with toxigenic ornithosis organisms, although Davis and Vogel[6] recovered the agent from chicks hatched from inoculated embryos. A previous attempt to isolate a disease agent from the progeny of a flock of birds naturally infected with an ornithosis agent also failed.[3]

Although the detection of ornithosis passive antibodies in the serums of turkey embryos or 1-day-old poults has not been reported previously, this observation is not surprising since transovarian passage of antibodies has been demonstrated in other poultry diseases.

TABLE 2—Results of Agent Isolation Attempts and Serologic Studies on Progeny of Ornithosis-Infected Turkeys

Material examined	Age in days	No. of specimens	Ornithosis agent isolation results	Serum ICF titer
Live embryos	7	24	Negative	.....
	14	2	Negative	.....
	16	2	Negative	.....
	21	5	Negative	.....
	23	14	Negative	1:4 (2 birds)
Dead embryos	2–4	153 (Pooled)	Negative	.....
	12	6	Negative	.....
	14	8	Negative	.....
	16	2	Negative	.....
	21	2	Negative	.....
	29	44	Negative	.....
Poults	1	13	Negative	9 negative 3 partial to 1:64 1 1:512
	38	8	Negative	All negative

The relationship between the disease agents isolated from turkeys and sparrows on the affected premises remains to be investigated. It is noteworthy that an ornithosis-like microorganism was also isolated from pigeons roosting near an infected turkey flock in a prior ornithosis epornitic in California.[3] In both epornities the source of the disease agent is entirely speculative, but the presence of similar organisms in intermingling wild and domestic avian species emphasizes the need for further ecologic investigations.

Summary

An ornithosis agent was isolated from turkeys of a breeder flock in California. The microorganism was described as a turkey ornithosis agent *(Miyagawanella ornithosis)* on the basis of morphologic, tinctorial, antigenic, and pathogenic characteristics.

Attempts to isolate an ornithosis agent by mouse-passage tests from 262 embryos at different stages of incubation and in 13 1-day-old poults hatched from eggs collected during the acute phase of the disease in the parent flock were unsuccessful. Serologic tests on the serums of 23-day embryos and 1-day-old poults from the same flock indicated that about 10 per cent showed indirect complement-fixation antibodies.

The source of the disease agent in the epornitic was not established; however, an unidentified microorganism causing lesions in mice indistinguishable from ornithosis lesions was isolated from the organs of sparrows collected on the affected premises after the epornitic occurred.

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Studies on Pathogenic Newcastle Disease Virus Contaminants in Newcastle Disease Wing-Web Vaccines

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SINCE 1952, increasing attention has been devoted to developing testing procedures that will ensure the safety, potency, and immunogenicity, as well as the purity, of biologic products used to prevent disease in animals. Johnson *et al.*,[22] in 1954, spotlighted the need for investigating the effectiveness of current minimum standards for vaccine testing, while Hanson [14] stressed some of the specific problems related to Newcastle disease vaccine. Contamination of live-virus vaccines with extraneous viruses has been reported by Zargar and Pomeroy,[29] Hofstad,[21] and Hanson.[14]

Federal minimum standards [11] for testing Newcastle disease (ND) vaccines require a safety test in which 12 birds, previously immunized against ND, receive ten times the field dose of the lot of vaccine under test; 3 intratracheally, 3 subcutaneously, 3 by skin scarification, and 3 intranasally. Three additional immunized birds must be maintained as contact controls. All of the inoculated birds are then observed for 14 to 21 days, during which time they must show no signs of ND or of any disease caused by extraneous agents.

The potency tests require the use of susceptible chickens no older than the youngest age recommended for application of the vaccine. At least 25 susceptible chicks are vaccinated, held in isolation for 14 to 21 days, and challenged with a lethal dose of pathogenic Newcastle disease virus (NDV).

Neither test seems adequate for detection of contamination of a vaccine by a strain of NDV that is more highly pathogenic than the vaccinal strain.

Two general classes of NDV are used in preparing live-virus ND vaccines: (1) the mild, or lentogenic, strains B1 and LaSota, which may be administered to chicks as young as 1 day old with little or no danger of unfavorable reaction; and (2) the mesogenic strains MK107 and Roakin, usually applied by wing-web puncture to chickens and turkeys over 4 or 5 weeks of age. The mesogenic strains seem to stimulate a longer-lasting and more solid immunity [21] than the lentogenic strains.[13] They are particularly useful for immunizing chickens or turkeys to be kept for egg production.[28]

Hanson and Brandly [13] devised an intracerebral pathogenicity index (ICPI) and a test [15] that have been used to detect possible contamination in the mild, or lentogenic, strains of ND vaccines. However, since both the mesogenic strains of moderate or high chicken pathogenicity and the highly pathogenic velogenic strains have similar ICPI, this test is of little value in detecting contaminants in wing-web vaccines.

Experiments designed to develop a test for pathogenic NDV contaminants in wing-web vaccines are reported in this paper. The mesogenic strains of vaccinal virus, when inoculated parenterally into susceptible chickens at least 6 weeks old, stimulate immunity with relative lack of pathogenicity or virulence.[3, 5, 7, 10, 12]

Preliminary work indicated that either of the mesogenic strains of NVD, N.J.-Roakin-1946 (hereafter referred to as Roakin) or Mass.-MK107 [L] (MK107), when inoculated intramuscularly (i.m.) or intravenously (i.v.) into susceptible chickens 8 weeks of age or older, induced an immune response without causing any clinical reaction, paralysis, or death. However, mixtures of either of these strains with the GB-Texas strain (GB), a widely used velogenic challenge strain of NDV, caused paralysis and death within ten to 12 days when inoculated i.m. or i.v.

These mixtures contained almost equal amounts of GB and Roakin, based on the 50 per cent embryo lethal dose (e.l.d.$_{50}$). Such massive contamination of vaccine is unlikely. Therefore, in most of the succeeding trials, either one or both of the vaccinal strains were used pure and were also used contaminated with about 1 p.p.m. of the GB strain. These were inoculated i.m. or i.v. and responses were observed.

To facilitate interpretation of observations, an intravenous or intramuscular pathogenicity index (IVPI) is proposed. The IVPI is a numerical expression of the pathogenicity of a virus and measures both severity of signs caused by the inoculum and the rapidity with which they develop. This index, based on observations for ten or 12 days

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postinoculation, is similar to the intracerebral pathogenicity index proposed by Hanson and Brandly[38] for characterizing different strains of NDV inoculated intracerebrally.

MATERIALS AND METHODS

Most of the experiments were conducted in California with susceptible chickens 4 to 12 weeks old. Some trials were done in experimental units at the University of Wisconsin. Equal numbers of susceptible chickens in wire-bottom cages were inoculated with 0.5 or 1.0 ml. of various dilutions of the pure vaccinal strains, the challenge strain (GB), or mixtures of GB and the mesogenic strains (Roakin or MK107, or both) under test. Observations were recorded for 11 to 13 days.

Facilities in California consisted of 5- x 10-ft. cubicles in which were placed seven or nine 18- x 18-inch wire-bottom cages, each holding 4 or 5 susceptible chickens. A separate unit was used for each virus strain or each mixture of strains under test. One cage of birds was kept as an uninoculated contact unit control for each cubicle or unit used (for each strain of virus or mixture of strains under test). Thirteen to 21 days after inoculations the principals and controls were challenged (i.m.) with the GB strain of NDV to assess their immune state. Blood samples were drawn at the beginning of each test and again just prior to challenge. In all cases, the hemagglutination-inhibition (HI) tests indicated susceptibility before inoculation. Birds susceptible to challenge at the end of the trial showed no HI response prior to challenge, while those resisting challenge with the GB strain had HI titers of 1:160 or higher.

The chickens used in the trials, all over 4 weeks old and less than 12 weeks old, were raised in isolation. In Wisconsin, they came from the University Poultry Husbandry Department; in California, from commercial hatcheries. In some cases at the University of California, Davis, chickens were placed in the experimental units two to three weeks before the trials; in all cases, control groups with the same history were examined concurrently with the principals, serologically and by challenge, to determine their susceptibility to NDV infection.

The strains of NDV used included Roakin, MK107, and GB, all obtained as frozen fluid from the NDV repository,[26] University of Wisconsin. Tryptose broth (Difco) was used as diluent for all virus dilutions.

The source of the MK107 strain was repository stock originally obtained from a vial of vaccine purchased on the open market. The Roakin strain used was Roakin-NJ-70903. The GB strain used had been grown in surviving cell cultures for one generation and was passed into 10-day embryos. Frozen embryo fluids were used as a source of virus for these trials.

Embryo fluids containing pure strains of virus were harvested, diluted, and mixtures of pure strains were prepared as needed.

Eggs obtained from commercial hatcheries were incubated nine, ten, or 11 days and then candled, marked, drilled, and used for virus passage and titrations. In California, the HI tests were run as described by Beach,[2] using the Montana strain of NDV as antigen. Fifty per cent end points were determined by the methods of Reed and Muench.[27]

Calculation of Intravenous Pathogenicity Index.—To arrive at a numerical index for the i.v. and i.m. pathogenicity of NDV, which are essentially the same, the severity of response was graded and a numerical factor assigned: 3 for death, 2 for paralysis, 1 for signs (dyspnea, rales, and malaise), and 0 for normal.

An illustration of IVPI calculation (table 1) includes observations of chickens for 11 days after inoculation with a mixture of the GB and Roakin strains of NDV. In terms of e.l.d.$_{50}$ the GB strain was about ten times as concentrated as the Roakin strain. Daily observations were recorded (*see* table 1). Any bird that died remained as a recorded observation during the entire test. The number of birds multiplied by the number of days observed equaled the total number of observation. Since 8 birds were used, the total number of observations was 88. The cumulative total of each recorded response (death, paralysis, signs, or normal) was multiplied by the numerical factor assigned for the specific response:

death × factor	21 × 3 = 63
paralysis × factor	15 × 2 = 30
signs × factor	5 × 1 = 5
	98 = coded number

The total of the results (coded number), divided by the total number of observations, equaled the IVPI:

$$\text{IVPI} = \frac{\text{coded number}}{\text{total observations}} = \frac{98}{88} = 1.11$$

The greater the pathogenicity of a particular virus strain or mixture of strains under test, the higher the IVPI. Theoretically, the IVPI may range between 0.0 and 3.0.

PROCEDURE AND RESULTS

A typical experiment is outlined (table 2); five cubicles were used. The virus strains tested were Roakin, mixtures of GB and Roakin, MK107, mixtures of GB and MK107, and GB. Controls and survivors were challenged with a lethal dose of the GB strain 19 days later. With the Roakin strain alone or with a mixture of 1 part of GB and 100,000 parts of Roakin, no pathogenicity was observed. The contact control birds remained susceptible and succumbed to challenge, even though they ate and drank from the same containers as inoculated infected birds in cages on either side.

With the MK107 strain and mixtures of MK107 and GB (500,000:1), there was

XUM

evidence of pathogenicity for MK107 alone. Interestingly, the mixture of GB (challenge) and MK107 (vaccinal) strains of virus evidenced less pathogenicity than did the vaccinal strain alone. All of the contact control birds acquired sufficient infection and developed sufficient immunity to have a significant HI titer and resist challenge 19 days after inoculation. This indicated spread of virus (presumably aerogenic) between inoculated and contact control birds and subsequent infection with immune response.

TABLE 1—Calculation of the Intravenous Pathogenicity Index of a Mixture * of the Roakin and GB-Texas Strains of Newcastle Disease Virus

Factor	Day of observation											Total
	1	2	3	4	5	6	7	8	9	10	11	
3 (death)	0	0	0	1	2	3	3	3	3	3	3	21
2 (paralysis)	0	0	0	1	2	2	2	2	2	2	2	15
1 (signs)	0	0	1	0	1	1	0	1	0	1	0	5
0 (normal)	8	8	7	6	3	2	3	2	3	2	3	47
Total observations												88

* Roakin, $10^{7.4}$ e.l.d.$_{50}$/ml. and GB, $10^{8.3}$ e.l.d.$_{50}$/ml.

The fifth group in this trial served as a titration of the bird pathogenicity of the GB strain given i.m. and permitted calculation of its IVPI and observation of its rate of spread.

This was one of the most extensive comparative trials conducted. Results in other experiments indicated, however, that the results were characteristic of the strains tested. The Roakin strain had the lowest IVPI and lethality for chickens over 4 to 6 weeks old; MK107 had slightly higher pathogenicity; and the GB strain was highly pathogenic. The results of challenge 19 days after initial virus inoculation demonstrates that the rate of spread from infected to uninjected birds nearby may be a characteristic of the virus strain; observations were similar in other trials. As in other trials, Roakin did not spread; GB spread slowly; and MK10 spread to all the contact birds, immunizing them. The lack of spread of infection between units was an indication of the effectiveness of the facilities and procedure used to prevent cross-infection between different units containing different strains or mixtures of strains.

Characteristics of three strains of NDV observed at the University of Wisconsin in 1955 and at the University of California in 1956 are summarized (table 3). Given i.m. or i.v., the three strains showed different pathogenicity: Roakin had virtually no pathogenicity, with an IVPI range of 0.0 to 0.22; MK107 had moderate pathogenicity, with an IVPI between 0.21 and 0.4 (in some

TABLE 2—Design and Results of Experiment with Newcastle Disease Virus—Intravenous Pathogenicity Index (IVPI) and Nineteenth Day Challenge

Virus inoculated (e.l.d.$_{50}$/ml.) *		Birds		Results	
		total	dead	IVPI	challenge, 19th day (dead/total)
Roakin	GB				
$10^{7.5}$	.....	8	0	0.0	0/8
$10^{6.5}$	.....	8	0	0.0	0/8
$10^{5.5}$	.....	8	0	0 0	0/8
None (control)		5	0	....	5/5
$10^{7.5}$	$10^{2.4}$	8	0	0.0	0/8
$10^{5.5}$	$10^{0.4}$	8	0	0.0	0/8
$10^{3.5}$	<1	8	0	0.0	0/8
None (control)	.....	5	0	....	5/5
MK107	GB				
10^{8}	.....	8	1	0.23	0/7
10^{7}	.....	8	2	0.31	0/6
10^{6}	.....	8	0	0.0	0/8
10^{4}	.....	8	2	0.40	0/6
None (control)	.....	5	0	.....	0/5
10^{8}	$10^{2.4}$	8	1	0.20	0/7
10^{7}	$10^{1.4}$	8	0	0.0	0/8
10^{6}	$10^{0.4}$	8	0	0.0	0/8
10^{4}	<1	8	1	0.16	0/7
None (control)	.....	5	0	.....	0/5
.....	$10^{2.4}$	8	8	2.22	.....
.....	$10^{1.4}$	8	7	1.75	0/1
.....	$10^{0.4}$	8	3	0.64	0/2
.....	<1	8	4	0.58	0/2
.....	None (control)	5	0	.....	3/5

* Intramuscular inoculation of 0.5 ml. per bird of each virus or each component in mixtures.

trials or subgroups, a range of 0.0 to 0.48); while GB (the challenge strain) evidenced pathogenicity with an IVPI of 0.8 when a dose of less than 10 e.l.d.$_{50}$ was inoculated per bird and an IVPI ranging from 0.58 to 2.38 at other dosages.

Observations made of the IVPI and the mortality following inoculation of mixtures of the GB strain and either of the two mesogenic strains under test are summarized (table 4). Except when the proportion of principals and of concurrent challenge of controls kept in separate cages (but in the same unit). Except for a single bird, all principals receiving an i.m. or i.v. inoculation of virus were immune to challenge.

Twenty-one of 25 contact control birds in experiments with the pure Roakin strain were still susceptible to challenge after 15 to 21 days' contact with inoculated birds. This indicated very little spread of this virus or resultant immunizing infection.

TABLE 3—Summary of Trials According to Dose of Pure Newcastle Disease Virus—Intravenous Pathogenicity Index (IVPI) and Lethality

	Newcastle disease virus strain					
	Roakin		MK107		GB	
e.l.d.$_{50}$ per ml.*	IVPI†	Lethality (dead/total)	IVPI†	Lethality (dead/total)	IVPI†	Lethality (dead/total)
<10	0.00	0/14	.....	.....	0.85	13/24
10^1	0.12	1/14	.....	.....	1.67	22/24
10^2	.....	.....	.....	.....	1.73	23/27
10^3	0.005	0/14	.....	.....	.....	.....
10^4	0.00	0/8	0.39	2/8	.....	.....
10^5	0.00	0/13	.....	.....	.....	.....
10^6	0.01	1/38	0.40	5/40	.....	.....
10^7	0.00	0/21	0.21	2/8	2.38	10/10
10^8	0.00	0/16	0.23	1/8	.....	.....

* Median dosage to nearest e.l.d.$_{50}$. † Weighted average of all birds inoculated within the range.

velogenic strain was at least 1 part in 10, there was no significant or characteristic increase in the IPVI due to admixture. While the IVPI of some mixtures of the Roakin and GB strains was greater than that of a similar concentration of Roakin alone, no mortality, paralysis, or death occurred in birds inoculated with 1 part of GB to 10,000 of Roakin. Thus, the admixture of GB to Roakin did seem to increase pathogenicity, but not in a uniform manner. However, adding GB to MK107 decreased pathogenicity below that for birds inoculated with similar dilutions of MK107 alone. This finding was consistent in four dilutions of MK107 tested, alone and as mixtures. The reason is not understood, unless rather large amounts of noninfective antigen in the mild strain were additive when mixed with the noninfective (but nonetheless antigenic) components of the dilutions of GB, thus blocking the actual infectiveness of the mixture. The IVPI of the strains tested alone and as mixtures are compared (table 5).

The characteristics of spread of the strains tested and of mixtures of each of the mesogens with the GB strain are summarized (table 6). The table is based on the results of challenge of the surviving

None of the control birds in experiments with mixtures of the Roakin and GB strains survived later challenge, indicating no spread of the mixed viruses.

In the experiments with the MK107 strain most contact control birds survived challenge, indicating rather rapid immunizing spread. None of the contact controls in tests with a mixture of MK107 and GB succumbed to challenge, indicating 100 per cent immunizing spread of infection.

Most of the contact controls in experiments with the GB strain alone died, indicating a rather slow spread of infection.

Discussion

Data presented here suggest that the i.m. inoculation of mixtures of virus containing both highly pathogenic velogenic NDV and moderately pathogenic mesogenic strains frequently used for vaccine production does not provide a criterion sensitive enough to detect the presence of small amounts of a pathogen in relatively large concentrations of the vaccinal virus.

There seems to be conclusive evidence that i.m. or i.v. inoculation of mixtures of pathogenic and vaccinal strains of NDV in normal birds (or even in birds concurrently

XUM

affected with infectious bronchitis) did not cause any serious signs of ND such as respiratory distress, paralysis, or death.

Observations made during these trials, not reported in detail here, suggest that extrinsic factors, such as coccidiosis or passive inmmunity, influence postinoculation response in a much more striking manner than does the presence of small quantities of a highly pathogenic virus in the vaccine itself.[8, 9, 24, 25]

Exploratory experiments subsequent to those reported here indicated that use of cage contact controls rather than unit contact controls did not greatly increase the sensitivity of the test for contaminants. Using wing-web inoculation of mixtures rather than i.m. inoculation also failed to increase the sensitivity of the test.

However, the inoculation of mixtures containing about 1 part of the GB strain to each million or 10 million parts of IVPI of 0.11 and 0.02 for MK107-GB and Roakin-GB mixtures, respectively.

The rate of growth of NDV strains is about equal;[20, 30] however, possibly because of differing growth potentials in embryo tissues or differing rates of nerve tissue invasion, embryo death times were 24 to 48 hours shorter for the GB strain than for MK107 or Roakin.

Multiplication of the GB (velogenic) component in the mixture may have continued, since 68 hours postinoculation the embryos were still alive, even though the mesogenic virus content reached maximum titer in 24 to 48 hours. The titer of the velogen may become more nearly equal that of the mesogen and overcome the interference manifested when there are proportionately large quantities of the less pathogenic mesogenic NDV. Thus, the component of the mixture present in low concentration (velogen) may increase sufficiently in a proportionate amount to become evident in i.m. or i.v. inoculation.

TABLE 4—Characteristics of Mixtures of Two Mesogenic Newcastle Disease Virus Strains with GB—Intravenous Pathogenicity Index (IVPI) and Lethality

e.l.d.so/ml.		Roakin		MK107	
Mesogen*	GB	IVPI	Lethality (dead/total)	IVPI	Lethality (dead/total)
10^1	10^1	1.08	4/8	.....	.....
10^1	$<10^1$	0.62	3/8	.....	.....
10^1	10^2	1.11	3/8	.....	.....
10^4	< 1	0.00	0/8	0.16	1/8
10^6	$<10^1$	0.00	0/20	0.06	0/20
10^7	10^1	.....	.....	0.00	0/8
10^7	1	0.13	2/8	.....	.....
10^6	10^1	0.10	2/8	.....	.....
10^6	1	0.14	1/8	.....	.....
10^5	10^2	0.00	0/8	0.20	1/8

* Concentration of the Roakin or MK107 strain in mixture with GB. When mixtures of indicated concentration were not used, no figures appear in corresponding IVPI or lethality columns.

Roakin or MK107 into 9-day chicken embryos and subsequent harvest of the virus-containing embryo fluids 68 hours postinoculation provided an indication of the presence of a highly pathogenic component. When chickens, 8 weeks old, were inoculated i.m. with such fluids containing MK107 and GB, 2 of 12 birds died before the twelfth day, and 2 additional birds were paralyzed. The IVPI for the mixture after embryo passage was 0.78. No unit controls were affected. In another group of 12 birds inoculated with Roakin-GB fluids, the IVPI was 0.17; 1 of the 4 room controls died on the ninth day of the test.

Two comparable groups of birds (given i.m. inoculations of the mixture of strains without previous passage through embryos) reacted in characteristic fashion, with an

Lockart and Groman[23] have demonstrated homologous interference by western equine encephalomyelitis virus in tissue culture pretreated with inactivated virus. Bang[1] demonstrated interference in embryos and chickens by inactive NDV, while Brandly *et al.*[10] showed protection by killed antigen. We suggest that intramuscular inoculation of mixtures of antigenic noninfective virus with living infective particles may interfere with virus invasion of host cells and prevent the development of signs of ND.

Additional evidence, however, may otherwise explain these results with fresh embryo fluids. It has been suggested that virus harvests from living embryos may contain

a much higher proportion of active or infective virus particles than do harvests from embryos that have either died or been killed by chilling before harvesting the fluids.

In all trials, except those involving passage of mixtures through embryos, the harvests were made from embryos that had been chilled to kill them or that had died in the incubator and were chilled after death and before harvest. In the trial involving passage of mixtures through embryos, passaged material for inoculation was harvested from embryos approaching death and immediately injected into susceptible chickens.

TABLE 5—Comparison of Intravenous Pathogenicity Index of Two Mesogenic Newcastle Disease Virus Strains Alone and Mixed with GB

e.l.d.$_{50}$/ml.		Alone		Mixed	
Mesogen	GB	Roakin	MK107	Roakin + GB	MK107 + GB
10	10^2	0.12	.....	1.11	.....
10	10	.....	.....	1.08	.....
10	<10	.....	.....	0.62	.....
10^4	< 1	0.00	0.39	0.00	0.16
10^6	<10	0.01	0.40	0.00	0.06
10^7	10	0.00	0.21	.....	0.00
10^7	1	.....	.....	0.13	.....
10^8	10^2	0.00	0.23	0.00	0.20
10^8	10	.....	.....	0.10	.....
10^8	1	.....	.....	0.14	.....

Other investigators may wish to extend these preliminary studies to clarify such results and further elucidate the pathogenesis and immunogenesis of ND.

The IVPI may be a useful characteristic to differentiate strains of NDV. The pathogenicity and the IVPI of GB, the velogenic strain used, were uniformly high. The IVPI of one of the two mesogenic strains used (Roakin) under the conditions of these trials was uniformly low, whereas that of the second mesogenic strain used (MK107) varied somewhat but in general was about midway between the velogenic strain (GB) and the mesogen of lowest pathogenicity. Whether tests of other strains of NDV would reveal characteristic IVPI remains to be explored.

Table 3 presents evidence that the pathogenicity of either the Roakin or MK107 strains for chickens older than 4 or 6 weeks decreases as the concentration of the virus inoculated is increased. It should be pointed out that, with the particular type

TABLE 6—Characteristics of Three Strains of Newcastle Disease Virus—Spread of Infection to Uninoculated Contacts and Surviving Principals

Birds	Challenge results* Total	Dead	Spread
Roakin-inoculated birds	113	0	Slight
Contact birds	25	21	
Roakin + GB-inoculated birds	67	1	None
Contact birds	18	18	
MK107-inoculated birds	47	0	Rapid
Contact birds	13	2	
MK107 + GB-inoculated birds	52	0	100%
Contact birds	13	0	Rapid
GB-inoculated birds	9	0	Incomplete
Contact birds	9	7	

* Thirteen to 21 days after primary virus inoculation, surviving principals and contact controls were given 10^5 to 10^6 e.l.d.$_{50}$ of the GB strain of Newcastle disease virus intramuscularly.

of inoculation practiced in these experiments (i.m. or i.v.), large quantities of antigen were inoculated. Some may have lost infectivity, but not the ability to induce immunity or to interfere with disease-causing NDV. With the velogenic strain (GB), however, the more concentrated the virus the higher the pathogenicity of the inoculated materials and the IVPI.

Another difference between the two mesogenic vaccinal strains studied was rate of spread, by a route presumed to be aerogenic. Similar differences were indicated in other work with other strains.[4, 5, 6, 7, 10] With few exceptions, the Roakin strain consistently failed to spread to contact birds with a lethal, paralytic, or even an inapparent immunizing infection. On the other hand, MK107 just as consistently spread from inoculated birds to contact birds in other cages in the same unit; it did not cause signs, paralysis, or death but immunized the chickens. This characteristic difference between the rates of spread of the two strains used for wing-web vaccine production was consistent throughout all the experiments and should be useful both as a differential characteristic of the strains and as a guide in initiating vaccination programs in areas where laying birds are not immune. Beach [3] reported that wing-web vaccine virus spread after inoculation to uninoculated birds, stimulating immunity. One strain of virus, MK107, was used in those trials.

In the trials reported here, either the i.v. or the i.m. route of inoculation was

XUM

used. However, in other trials, use of the same strains of virus by wing-web inoculation resulted in similiar differential characterization. Examination of additional strains of NDV under similiar circumstances is indicated in order to characterize their potential for spread, as well as their IVPI.

In one of the experiments in which decreasing doses of the Roakin strain were used, the results of challenge at the termination of the trial suggested that the smaller amounts of i.m.-inoculated virus had a slightly greater tendency to spread than the more concentrated i.m. inoculations and, likewise, induced less immunity. The groups of birds receiving the lowest dose of the immunizing virus developed slight transitory respiratory signs following i.m. challenge with the GB strain, whereas those receiving larger amounts of the immunogen (Roakin) did not.

Thus, though the primary purpose of the investigations failed, *i.e.*, developing a useful test for ND pathogens in ND wing-web vaccines, two additional differential characteristics have been established for two mesogenic strains, Roakin and MK107. The rate-of-spread characteristic seems more consistent than the IVPI but, in addition to the differences described by Hanson *et al.*,[16,17,19] both are useful for characterizing these strains.

The observation that the admixture of the GB with the MK107 strain reduced the severity of the response when inoculated i.m. or i.v. has not been explained. The effect may be due to the presence of large amounts of noninfective antigen in the material and a blocking of disease-causing infection. Additional investigations are needed to clarify this point.

The information developed in these trials support the proposal of Brandly *et al.*[10] that use of inactivated virus followed by live virus after two weeks would provide a useful vaccination. We suggest that concurrent i.m. vaccination with inactivated virus plus live mesogenic vaccinal virus also merits consideration. It seems apparent that MK107 might be useful as an airborne vaccine in addition to its present use by the wing-web route.[26]

SUMMARY

In an attempt to develop satisfactory methods for detecting viral contaminants in Newcastle disease wing-web vaccines, tests were made on the pathogenicity of the two most commonly used mesogenic strains, N.J.-Roakin-1946 (Roakin) and Mass.-MK107 [L] (MK-107), alone or contaminated with the GB-Texas (GB) strain, a highly pathogenic challenge (velogenic) virus. Injections were made intravenously (i.v.) or intramuscularly (i.m.) in chickens over 6 weeks of age.

A numerical expression of the pathogenicity of strains, the intravenous pathogenicity index (IVPI), is proposed. This is a measurement of the severity of postinoculation signs and the rapidity with which they develop. The IVPI ranges from 0.0 to 3.0 (nonpathogenic to highly pathogenic); this provides a numerical expression of the relative pathogenicity of NDV when inoculated i.v. or i.m. in susceptible chickens, 6 weeks old or older.

Of the strains tested under the conditions described, GB had the highest pathogenicity (IVPI, 0.85–2.38), Roakin had the lowest (IVPI, 0.0–0.12), while the pathogenicity of MK107 (IVPI, 0.21–0.40) was between that of Roakin and GB. The rate of spread from inoculated birds to contact controls was characteristic of the strain used—Roakin spreading hardly at all, GB spreading slowly, and MK107 spreading rapidly and stimulating immunity in the contact control birds. The concentration of virus and the strain used affected the IVPI, the rate of spread, and the immune response.

Intravenous or i.m. inoculation of mixtures of either mesogenic vaccinal strain of NDV (Roakin or MK107) with a highly pathogenic velogenic strain (GB) showed that the less pathogenic strain interfered with the effect of GB. Hence, i.m. or i.v. inoculation of susceptible 6-week-old chickens does not provide a satisfactory gauge of contamination of ND wing-web vaccines.

The possible application of the information to vaccine use, manufacture, and testing is discussed, as are the implications concerning interference, importance of extrinsic factors, and immunologic significance.

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A Study of the Oocystic Sporulation of Some Avian and Mammalian Eimeria Species Under Simulated Egyptian Summer Conditions, with Special Reference to the Cycle of Oocyst Production of *Eimeria Maxima*

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As a disease of poultry, coccidiosis ranks next in importance to fowl plague, Newcastle disease, and fowl cholera. During the past few years, several workers [1-12] have studied the morphology, distribution, periodicity in oocyst output, and normal sporulation of the different species of Eimeria and Isospora. However, adequate data is lacking on the biology of these species, particularly the sporulation of the oocysts under the variable conditions of temperature and humidity.

The fact that a bird might harbor Eimeria infection, but not pass oocysts at times, has not been established. Such data would be significant in diagnosis, as the cessation in the elimination of oocysts makes it difficult to determine whether birds are free from parasites. Knowledge of oocyst production is also an important factor in determining the therapy of various related diseases with similar clinical signs, as it is a gauge of parasite activity within the host. Furthermore, this information would shed light on many other valuable hypotheses of the host-parasite relationship.

Abnormally rapid sporulation of oocysts in cultures prepared for chemotherapy experiments was noticed by us during the summer of 1958. Also, repeated daily fecal examinations of experimentally infected chickens showed periodic disappearance of oocysts. To explore these phenomena, a systematic study was made of the stages of oocystic sporulation, to correlate its speed and the viability of oocysts under local (Giza) summer conditions, then was repeated on available mammalian and avian materials. The various stages of the cycle of oocyst production of *Eimeria maxima*, with special reference to its negative periods, were also experimentally determined, since reference to these points seemed not to have been previously established.

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Material and Methods

The material for this study consisted of oocysts obtained from avian and mammalian hosts at the Faculty of Agriculture, Giza. In chickens, inflammation at the middle and terminal regions of the intestine was demonstrated to be the result of *E. maxima* infection. Eimeria species studied were *E. maxima*, *E. faurei* (from sheep), *E. perforans*, *E. media*, and *E. magna* (from rabbits).

The infected intestinal contents were washed several times, then suspended in 2.5 per cent potassium bichromate solution to which a few crystals of pencillin sodium had been added to minimize bacterial growth. This material was then distributed in Petri dishes and allowed to sporulate in a room with glass windows at an atmospheric temperature of 29 to 31 C., and a relative humidity of 52 to 55. Cultures were examined once or twice daily until the end of complete sporulation. The infectivity of the microscopically sporulated oocysts was measured by inoculation of uninfected controls.

Experimental chickens were about 6 to 8 weeks old, of a local breed, and Eimeria-free. They were kept in clean, screen-bottomed wire cages to prevent the possibility of any adventitious infection. In this way, it was possible to obtain correct results during the study of the oocystic output in hosts inoculated with single infection. Control birds were usually kept under the same climatic and nutritive conditions.

In cross infection, 1 cc. of completely sporulated oocyst suspension was inoculated with a graduated pipette, directly into the crops of 16 chickens. The inoculum contained an estimated 66,500 oocysts, as indicated by daily progressive sample counts, using the McMaster slide technique.

To obtain more accurate and uniform data on the daily average number of oocysts, fecal examinations were carried out at a fixed midday time, when the peak of oocyst production was usually reached.[5] These counts were made beginning the day after infection and were repeated for 36 successive days.

Results

Oocystic Sporulation.—Under the previously mentioned climatic conditions, hourly or daily culture samples showed that in *E. maxima* no zygotic division inside the oocyst occurred before 48 hours. At this time, most of the oocysts showed development to the sporoblast stage only, while the sporocyst formation was reached in 72 hours after defecation. Complete sporulation in all cultures appeared to have been reached for most living organisms in approximately 96 hours. This was in contrast with Becker's observations on *E. maxima*,[1] and those of Edgar [10] on *Eimeria tenella*, recording oocystic activity after 30 hours at 28 C.

Surprisingly, oocysts of *E. faurei* of sheep exhibited rapid development after only 24 hours, when most of them reached the sporocystic stage. By 48 hours, complete sporulation was clearly evident in all culture dishes. In the case of the three species from the rabbit, *E. perforans* demonstrated sporulation similar to ovine *E. faurei* with 70 per cent of the encysted zygotes showing activity until the sporocystic stage at 24 hours. Sporozoite formation of 90 per cent was reached by the end of 48 hours. At the same time, no sign of activity was seen in either *E. media* or *E. magna* oocysts except after 48 hours, when 70 per cent of the former developed to the sporocyst stage, reaching maturity by the end of the 72 hours. *Eimeria magna* required up to 96 or 108 hours to sporulate.

This variable degree of activity in sporulation among the species studied might be partly attributed to the degree of the inclusive zygotic activity, to the characteristics of the oocyst wall, or to the permeability of the micropyle membrane and the nature of its cap.

Therefore, it seemed reasonable to conclude that, when the atmospheric temperature is comparatively high, oocystic sporulation in Eimeria species probably requires more variable periods of time than those reported previously.

Cycle of Oocystic Production.—In agreement with the findings of Becker,[1] repeated daily fecal examinations of the 16 experimentally infected chickens revealed that the prepatent period was approximately six days in all birds. Representative oocyst counts are shown (fig. 1).

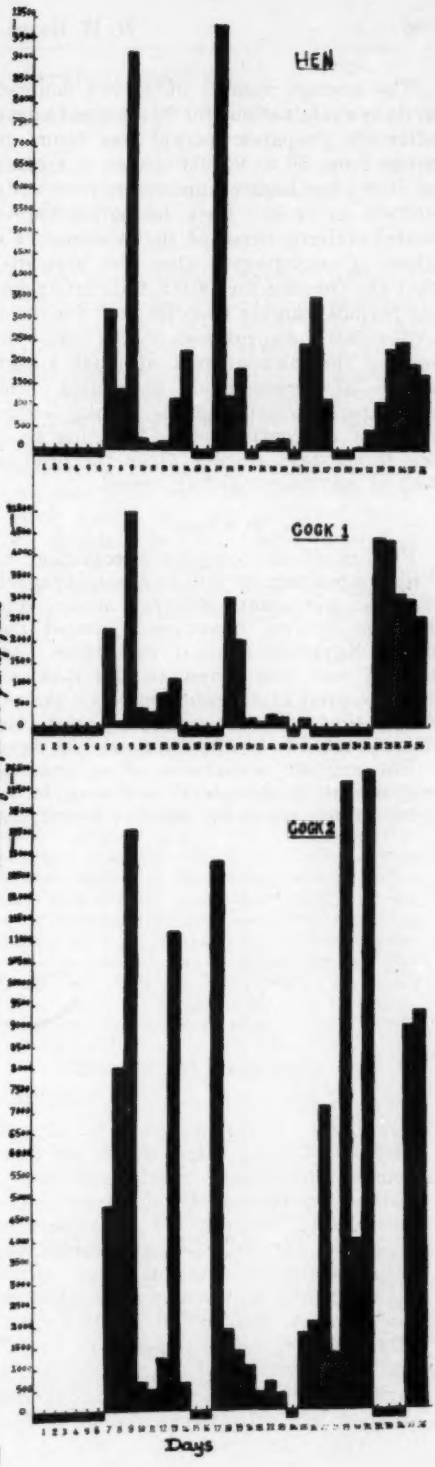

Fig. 1—Variation in the number of oocysts of Eimeria maxima in feces collected daily for 36 days from 3 experimentally infected chickens.

The average number of oocysts counted in daily examinations for 30 successive days after the prepatent period was found to range from 50 to 91,800 oocysts per gram of feces; the higher numbers were usually noticed in cocks. Each histogram represented striking proof of the existence of a phase of encystment. They also indicated that the oocystic formation falls into regular periods ranging from three to five days. Peaks were approximately the same in most of the chickens used, although a high degree of agreement is impossible. The data also demonstrated the regular occurrence of a negative period, ranging from one to six days, during which the production of oocysts completely ceased.

SUMMARY

Studies of the oocystic sporulation of *Eimeria maxima*, as well as *Eimeria faurei*, *Eimeria perforans*, *Eimeria media*, and *Eimeria magna*, have been carried out under Egyptian summer conditions. Attention was also given to the cycle of oocystic production, represented by that of *E. maxima*. The incubation period was demonstrated to be approximately six days.

The regular occurrence of a cycle of encystment, with special reference to its intermittent, regularly negative period, has been experimentally investigated for the first time. The duration of the cycle has been calculated to range from three to eight days; the negative period is from one to six days.

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Studies of the Effect of Oxytetracycline on Chicken Spermatozoa

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IN A PREVIOUS PAPER,[13] the effects of a number of antibiotics on motility, fertility, and bacterial count of chicken semen were reported. The combination of dihydrostreptomycin (DSM) with either penicillin or oxytetracycline (OTC) was the most effective in reducing bacterial number; however, only the latter combination resulted in improvement in fertility. The difference in the results with fertility raised the question as to whether a bacteriostatic effect was involved, since both combinations were equally effective in this regard.

It is known that the tetracyclines act as chelating agents,[9] and one possible explanation of the results cited is the removal of heavy metals from the diluted semen by the process of chelation. Although the heavy metals have not been studied with chicken sperm, it has been shown [11] that several of them are toxic to mammalian spermatozoa. Furthermore, it has been observed that the chelating agent, ethylenediaminetetracetic acid (EDTA), when added to sea water, greatly lengthens the life span of sea urchin sperm [5,6] and starfish sperm,[2] an effect which was attributed to the chelation of the heavy metals of sea water. Several other chelating agents have proven to be toxic to mammalian sperm [10] at 0.1 mM; this toxicity has subsequently been found to be decreased in the presence of cobalt but increased in the presence of several other metals.[12] Utida *et al.*[7] found the inhibition of apyrase activity of starfish sperm by zinc to be prevented by EDTA.

Accordingly, experiments were conducted to test for chelation by OTC. First, fertility experiments were undertaken in which OTC and DSM were added to the diluent both separately and in combination. The OTC alone has been shown to be ineffective against bacteria, but DSM either in combination with OTC or alone is effective.[13] If the effect of OTC and DSM on fertility is due to antibacterial action, it would be expected that fertility would be greater with the DSM. Another possible basis for the improved fertility with OTC and DSM is the presence of citric acid in the preparation of OTC that was used. Therefore, amounts of citric acid were used in the fertility tests equivalent to the amount present in the levels of OTC employed. Citric acid is known to be present in large amounts in the semen of certain mammals and to be utilized by their sperm.[3]

EXPERIMENTAL PROCEDURE

Experimental conditions, material, and stock were similar to those reported previously.[13] In all cases, except where stated otherwise, the compounds used were added to the phosphate buffer before dilution and storage. In the fertility tests, semen was diluted 1 to 10 and concentrated by centrifuging at 1,200 R.C.F. for ten minutes, supernatant fluid was poured off, and the packed cells were diluted to the original volume before dilution with a buffer containing 4 mg. per milliliter of fructose. Motility scores obtained were multiplied by 12 to eliminate the use of fractions, and the spermatozoa were stored for one day only at 10 C.; KCl and $MgCl \cdot 6H_2O$ were added to the buffer as isotonic solutions containing 13.6 and 25.3 Gm. per liter, respectively.

RESULTS AND DISCUSSION

FERTILITY

Results of the four fertility trials conducted are shown (table 1). In two cases, the centrifuge tubes broke and the data for those particular treatments are missing. The results of the separate trials were quite variable, as is often the case with fertility tests. However, some interesting observations can be made by comparing the average fertility in the four trials for the different treatments. The fertility with the OTC (90 μg./ml.) and DSM was considerably higher than with the buffer alone, which is in agreement with previous re-

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sults.[13] The results from treatments with DSM and with citric acid at two levels were nearly identical to those with buffer alone. Fertility was improved at both levels of OTC whether or not it was combined with DSM. There was a tendency for use of the higher levels of the OTC to result in slightly better fertility than the lower levels.

These results indicate that the beneficial effect on fertility is due to the presence of OTC, and is not related to bacteriostatic action of the antibiotics used, or due to citric acid. Hatchability of eggs fertilized with these spermatozoa was unaffected by the treatments employed.

TABLE 1—Fertility in Semen Treated with Different Levels and Combinations of Antibiotics and Citric Acid Added to a Phosphate Buffer and Stored 30 to 31 Hours

Compound*	Percentage of fertility in each trial (first week after insemination)†				
	1	2	3	4	Average**
None	17	43	79	26	41
OTC (90 μg./ml.) + DSM (900 μg./ml.)	49	56	75	59	60
OTC (180 μg./ml.) + DSM (900 μg./ml.)	....	41	83	75	64
DSM (900 μg./ml.)	19	39	73	38	42
OTC (90 μg./ml.)	65	44	71	49	57
OTC (180 μg./ml.)	61	59	86	68	69
Citric acid (0.14 mg./ml.)	47	29	59	34	42
Citric acid (0.28 mg./ml.)	49	22	....	41	45
Unstored, undiluted control	78	84	76	74	78

OTC = oxytetracycline; DSM = dihydrostreptomycin.
* Level in diluted semen. † Average of 11 hens and 57 eggs (range = 39–71). ** Adjusted for missing value.

MOTILITY

Motility tests were conducted with a wide range in concentration of OTC and EDTA to compare the effect of OTC with that of a known chelating agent. Motility was markedly reduced at the highest level of OTC used and the three highest levels of EDTA used, and was unaffected at the lower levels (table 2). Difficulty was encountered in dissolving the OTC at levels higher than 900 μg. per milliliter.

Additional motility tests were conducted to determine if OTC would act to reduce the harmful effect of heavy metals at toxic levels. An attempt was made to use all of the metals employed by White,[10] but unfortunately none of them would dissolve in the phosphate buffer, even when one part of the buffer was diluted with 19 parts of saline solution (9.9 Gm. NaCl/l.). It was possible, however, to dissolve nickel ($NiSO_4 \cdot 6H_2O$) in a diluent containing equal parts of phosphate buffer and saline solution. This metal is known to be chelated both by OTC [9] and by EDTA.[4] Also, varied levels of nickel were used singly and in combination with OTC or EDTA, using the latter two at the highest levels which did not reduce motility (table 3). Motility was impaired only at the highest level of nickel used. The addition of EDTA to nickel at this level did not improve motility, but there was a slight improvement when OTC was added. Trials 7, 8, and 9 consisted of two samples per treatment, whereas trials 10 and 11 consisted of five per treatment. Statistical analysis revealed that the improvement of motility when OTC was added to nickel was not significant in trial 10 but highly significant in trial 11. Some data were also obtained using a higher and slightly toxic level of OTC (450 μg./ml.). These results indicate very little effect of the different combinations employed. Precipitation was observed in all diluents containing nickel combined with OTC or EDTA except at the lowest two levels of nickel used.

TABLE 2—Motility Scores of Sperm Treated with Various Levels of Oxytetracycline (OTC) and Ethylenediaminetetracetic Acid (EDTA) and Stored One Day at 10 C. (Trials 5 and 6)

mM*	OTC		EDTA	
	μg./ml.*	Motility score	μg./ml.*	Motility score
None	.....	32	.....	32
0.00062	.....	....	0.18	28
0.0031	1.44	33	0.9	29
0.016	7.2	33	4.5	33
0.078	36.0	29	22.5	29
0.39	180.0	33	114.0	10
1.95	900.0	2	569.0	6
9.74	.....	....	2,844.0	7

* Level in diluted semen.

Thus, results are not consistent regarding action of OTC in reducing the harmful effect of nickel on motility. An improvement in motility was always present, but was rather small in some cases. In the case of a toxic level of OTC (0.98 mM), the effect observed was not similar to that found by Utida *et al.*[7] in which a harmful level of a metal and of a chelating agent was found not to be harmful when the two compounds were combined. There does not appear to be any other information on the harmful effect of nickel, although it has been reported [10] that nickel reduced the toxicity of o-phenanthroline.

Another aspect of the problem concerned the toxic effect of high levels of the OTC and EDTA. If this is an effect of chelation, it would seem likely that it is due to the removal of certain beneficial metals such as magnesium, as has been previously suggested.[7] It is known that, both with OTC[1] and EDTA,[4] magnesium is bound less strongly than most of the divalent cations studied and would be expected to be one of the last bound by chelation. Therefore, a motility test was conducted in which the high levels of EDTA and OTC were employed with and without added magnesium or potassium. The latter two cations were used at levels within the range found by Wales and White [8] to enhance motility. The results (table 4) show clearly that potassium is not involved, since OTC or EDTA plus potassium are as toxic as the chelating agent alone. However, the addition of magnesium resulted in motility scores higher than that of the buffer control but somewhat less than that of the buffer with magnesium. The marked improvement of motility with magnesium alone compared to the buffer is also of interest and is in essential agreement with other data.[8] Magnesium was also added to sperm already inhibited by high levels of OTC or EDTA; in some cases, this resulted in reversal of inhibition.

Thus, the inhibitory action of OTC or EDTA at high levels is probably due to removal of magnesium and possibly other cations from solution. Similar results have been

TABLE 4—Motility Scores of Sperm Treated with Buffer Containing Oxytetracycline (OTC) and Ethylenediaminetetracetic Acid (EDTA) at Levels Previously Shown to Reduce Motility, Both Singly and Combined with Mg^{++} or K^{+} (Trials 12 and 13)

Compounds*	Motility score
None	32
OTC (1.95 mM)	11
OTC + KCl (9 mM)	6
OTC + $MgCl_2 \cdot 6H_2O$ (9 mM)	34
EDTA (0.39 mM)	15
EDTA + KCl (9 mM)	16
EDTA + $MgCl_2 \cdot 6H_2O$ (9 mM)	42
KCl (9 mM)	37
$MgCl_2 \cdot 6H_2O$ (9 mM)	46

* Level in diluted semen.

TABLE 3—Motility Scores of Sperm Diluted with a Buffer Containing Nickel, Oxytetracycline (OTC), and Ethylenediaminetetracetic Acid (EDTA), Singly and in Combination

Levels of compounds*			Motility scores			
$NiSO_4 \cdot 6\ H_2O$ (mM)	OTC (mM)	EDTA (mM)	Trial 7	Trials 8 & 9	Trial 10	Trial 11
.....	.....	.....	46	44	46	46
0.018	.....	.....	38	....	....	....
0.18	.....	.....	38	40	....	....
0.45	.....	.....	....	40	....	....
0.9	.....	.....	....	35	....	....
1.8	.....	.....	16	23	18	14
.....	.....	0.078	44	....	....	....
0.018	.....	0.078	48	....	....	....
0.18	.....	0.078	44	....	....	....
1.8	.....	0.078	12	....	....	....
.....	0.39	.....	36	39	39	42
0.018	0.39	.....	24	....	....	....
0.18	0.39	.....	34	39	....	....
0.45	0.39	.....	....	33	....	....
0.9	0.39	.....	....	34	....	....
1.8	0.39	.....	20	31	23	33
.....	0.98	.....	....	20	....	....
0.18	0.98	.....	....	23	....	....
0.45	0.98	.....	....	27	....	....
0.9	0.98	.....	....	26	....	....
1.8	0.98	.....	....	23	....	....

* Level in diluted semen.

found with sperm apyrase.[7] Results of other workers showing that certain chelating agents are harmful could very likely be due to the particular level used; the level used by White[10] was between the highest level found to be nontoxic and the lowest level found to be toxic in results reported here with EDTA. Utida *et al.*[7] found EDTA to be harmful at 0.1 mM, which is not in disagreement with the results found here, whereas Tyler[6] found EDTA to be beneficial at 0.1 mM but harmful at 1.0 mM. This difference may have been due to the large amount of heavy metals present in sea water. The presence of heavy metals could affect the toxicity curve for EDTA, since it has been shown[7] that as much as 3.3 mM of EDTA are needed before apyrase activity is even slightly reduced in the presence of calcium, whereas without calcium the activity was reduced at levels as low as 0.1 mM.

CONCLUSIONS

The results as a whole indicate that the most plausible explanation of the beneficial effect of OTC on fertility is chelation of heavy metals. Reasons for this conclusion are the failure of bacteriostatic potency or additional citric acid to account for the effect on fertility, and the tendency for the OTC to reduce the toxicity of nickel. The identity of harmful metals normally involved is unknown; it has been suggested[5] that copper is involved with sea urchin sperm, and the data with starfish sperm[7] strongly suggest the involvement of zinc.

SUMMARY

Chelation has been tested as a possible explanation for the beneficial effect on fertility of oxytetracycline (OTC) in combination with dihydrostreptomycin (DSM). It has been shown that fertility is improved whether OTC is or is not combined with DSM, and is not improved with DSM alone. This improvement in fertility does not appear to be related to the bacteriostatic potency of the antibiotics tested. It has been shown that OTC and ethylenediaminetetracetic acid reduce motility at higher levels. This reduction is markedly reduced by the addition of magnesium. Nickel reduced motility only at the highest level used (1.8 mM). This reduction was mitigated to some extent by the addition of OTC.

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Spontaneous Cataracts in Turkeys

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SPONTANEOUSLY OCCURRING cataracts have been observed in chickens.[1,5,6] However, similar lesions apparently have not been reported in the turkey. During the past several years we have been interested in the spontaneously occurring cataracts in the chicken,[6] the cataracts in turkey embryos obtained from hens deficient in vitamin E,[3,4] and the degeneration of the lens in chick embryos injected with dinitrophenol.[2,7] While these studies were in progress, 7 turkeys developed spontaneous cataracts. Observations on this group of turkeys are reported.

TABLE 1—Turkys with Normal Eyes

No. of turkeys	Breed	Age first examined (days)	No. examinations	Age at death (days)
10	BSW	22–26	1	22–34
8	BBB	26–36	1	26–36
10	BBB	40–57	1	46–111
7	BBB	40–57	8–13	257–387
9	BBB	61–69	1–9	62–146
4	BBB	90	4–14	195–462
8	BSW	118–169	1–2	122–169
2	BBB	135	8	254–257
1	BSW	221	11	558

BSW = Beltsville Small White; BBB = Broad Breasted Bronze.

MATERIALS AND METHODS

Fifty nine normal turkeys (table 1) and 7 with cataracts (table 2) have been studied. Those with cataracts were Broad-Breasted Bronze turkeys, except for 2 that were Beltsville Small Whites.* The dams had been fed a complete practical ration and the poults were fed a complete synthetic diet until shipped to Galveston for additional study and observation. Six of the birds were shipped at 14 days of age and the seventh bird was shipped when 35 days old. All birds were fed a standard commercial ration while under observation at Galveston. Food and water were available at all times. The birds were housed in batteries and in small pens.

The eyes were examined by using a standard type of dissecting microscope and a small spot light. The frequency of the examinations varied with different turkeys. The day the cataracts were first seen and the length of time these birds were observed are recorded (table 2). The time and number of examinations and the age at death of the 59 control turkeys are also shown (table 1).

TABLE 2—Summary of Data on Turkeys with Cataracts

Turkey No.	Sex	Age cataract first observed (days)	Death		Lens involvement		Crystals		Detached retina
			Age (days)	Weight (kg.)	Right	Left	Right	Left	
31586	....	35	100*	1,320 g.	++	+++	0	0	0
1127	F	245	291	.....	0	+	0	0	0
1128	M	89	692	11.3	++++	+++	+	+	+
1129	M	108	337	12.7	+	+	0	0	0
1130*	M	90	297	.....	+	+	0	0	0
1131	F	219	863	6.8	+++	++	0	0	+
1132*	F	90	766	8.0	++++	+	+	+	0

* Turkey that died (all others were killed).

RESULTS AND OBSERVATIONS

The first cataract observed was in turkey 31586 when it was 35 days old. The cataract in the left eye on day 40 was easily seen macroscopically (fig. 1). When observed microscopically, small crystals radiated outward from the central mass. The cataract in the right eye was similar but smaller than that in the left eye. No significant changes were observed in the lenses during the 65 days under observation. Sev-

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* All birds were hatched at the poultry farm at Texas A. & M. College, College Station.

XUM

Fig. 1—Cataract in left eye of turkey 31586, 40 days old.

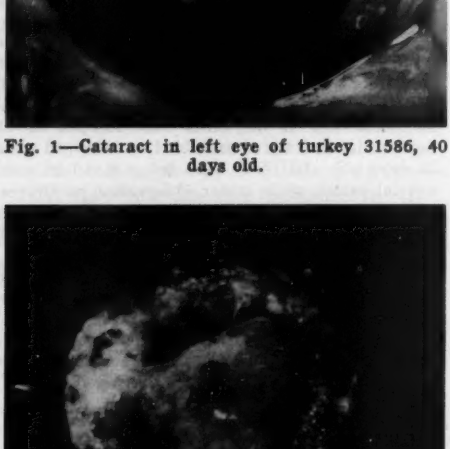

Fig. 2—Posterior view of the lens of turkey 1128 showing the cataract (A) and the crystals (arrow).

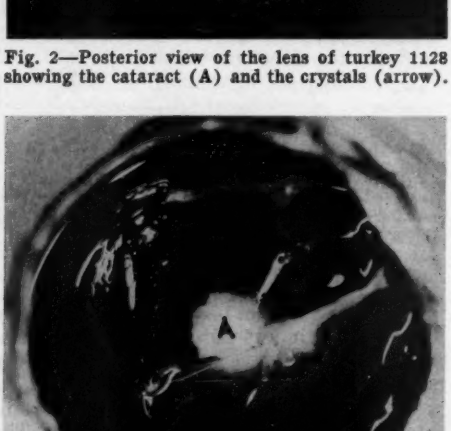

Fig. 3—Posterior view of the lens (A) and the detached retina (B) of turkey 1128. The bird was killed when 692 days old.

eral days before this bird was killed, he appeared weak and apparently had some difficulty in breathing. A complete necropsy was performed and all tissues except the eyes were studied microscopically. No significant lesions were observed in the viscera.

The other 6 turkeys that developed cataracts were hatched on Jan. 31, 1956. The eyes of turkey 1128 were first observed to have a cataract on day 89. By day 107, approximately three fourths of each lens was opaque. On day 237 this turkey appeared to be blind. He showed no indication of sight when a hand was moved in the area of his head. On day 321 numerous small crystals on the surface of both lenses could be observed, but no changes in the anterior chamber. The opaque area in the lens and the small crystals persisted. This bird retained a normal weight for a year, although he appeared to be completely blind.

Pathological changes were studied only in the eyes of turkey 1128. The left eye was fixed immediately in formaldehyde and subsequently observed with the dissecting microscope. The lens was opaque and many small crystals were present on both the inner and outer surfaces (fig. 2). These crystals apparently were the same as had been observed during the past year. The retina in the right eye was detached (fig. 3). The lens from the right eye was removed without fixation and teased out in distilled water. Numerous small crystals were present in the lens (fig. 4). When examined under polarized light, these crystals were yellow and when rotated 90 degrees they were blue. The right eye without the lens was fixed in a 4.0 per cent solution of formaldehyde, and paraffin sections were prepared. The choroid was infiltrated by a moderate number of lymphocytes. The anterior surface of the choroid in the area where the retina normally belongs was covered by a layer of fibrous tissue.

A small opaque area was present in each lens of turkey 1130 on day 90. These changes persisted with little variation until death on the 297th day. The liver was large and contained many focal, grayish red abscess-like areas. The kidneys were large and yellowish brown. No additional macroscopic changes were observed. Neither the viscera nor the eyes were studied histologically.

Cataracts were first observed in turkey 1132 on day 90. The opaque area in the right eye was several times larger than that in the left. The cataracts in each lens gradually increased in size. After eight months, approximately three fourths of the right lens was opaque, and a year later the bird apparently was completely blind in this eye (fig. 5). Long needle-like crystals (fig. 6) and small crystals similar to those shown in turkey 1128 (fig. 3) were present in this eye. The former crystals may have been in the posterior chamber and the latter in the lens. On the 766th day this turkey was found dead. A degenerated egg was found in the abdominal cavity. The lens from the right eye had crystals that were similar to those in turkey 1128 (fig. 4). No histological studies were made of either the eyes or the viscera.

Cataracts were first observed in both eyes of turkey 1129 on the 108th day. They were small and changed little during the time this bird was under observation. On day 337 he appeared to be sick and was killed. The liver was large and had small focal abscesses. Multinucleated cells were present about the periphery of these areas of necrosis. A large amount of yellow-brown pigment, resembling hemosiderin, was present in the spleen and liver. The eye was not studied histologically.

Cataracts were first observed in both eyes of turkey 1131 on the 219th day; they progressively increased in size. This turkey appeared normal on day 863, at which time she was killed. Approximately one third of the right lens was opaque. Small crystals were present in the right eye similar to those described in turkey 1128. The retina was detached in the right eye and a layer of fibrous tissue was present over the choroid in the affected area. Bony metaplasia was present in the choroid. There were focal areas of cystic degeneration in the lens. Many of the epithelial cells in the right lens were large and degenerated (fig. 7). Foci of proliferated cells occurred in the lens adjacent to the capsule (fig. 8).

A small opaque area was present in the left lens of turkey 1127 on day 245. This opacity changed little during the time this bird was under observation. The right eye seemed normal. This bird was killed on day 291, but no studies were made of the eyes or viscera.

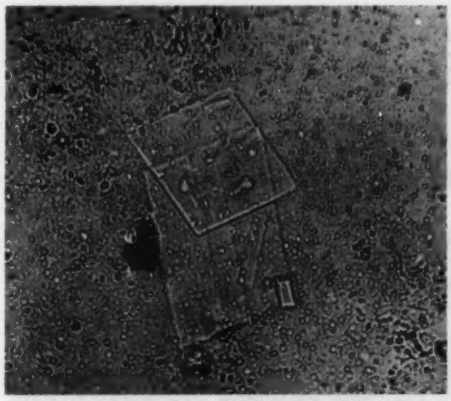

Fig. 4—Crystals in the degenerated lens of turkey 1128 as shown with polarized light; x 260.

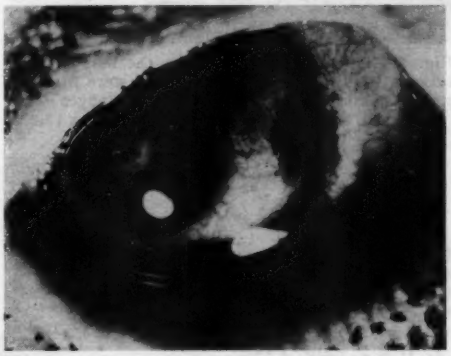

Fig. 5—Cataract in the right eye of turkey 1132 on day 707. This cataract was first observed when the turkey was 90 days old and persisted until death on day 766.

Fig. 6—Crystals from the right eye of turkey 1132. Polarized light; x 522.

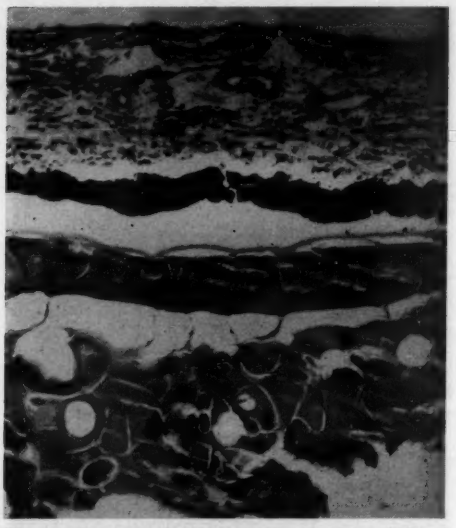

Fig. 7—Degenerative changes in the lens (A) of turkey 1131, 863 days old. Changes were first observed on day 219. No significant changes were observed in the iris (B). H & E stain; x 150.

anterior chamber or in the iris, such as was recently described in chickens with cataracts.[6] In the 2 turkeys with the more extensive cataracts, the retina was detached.

The lens changes in these turkeys are similar to those recently described in chickens with lymphomatosis.[6] In the chicken, cataracts occurred in birds with iritis; however, iritis was not observed in these turkeys. Furthermore, the turkeys developed normally. Detailed histological studies unfortunately were not made on the eyes or viscera of each of these 7 turkeys.

Since the turkeys that developed cataracts were in contact with chickens infected with the virus of lymphomatosis, it seems most likely that the virus was transmitted to them from the chickens. Experiments are in progress to prove this hypothesis. The similarity of the lens degeneration in these chickens and turkeys is obvious.

SUMMARY

Spontaneous cataracts are described in 7 turkeys. This lesion was first observed in birds less than 3 months old. The lens changes frequently progressed until vision was markedly decreased. A detachment of the retina occurred in 2 of the 7 turkeys. There are indications that the etiological factors in this lens degeneration may be the same as that in chickens with lymphomatosis.

Fig. 8—Local proliferation of cells immediately below the capsule (A) in the same lens (turkey 1131) as shown in figure 7. H & E stain; x 440.

The pathological changes observed in the lenses of these 7 turkeys are summarized (table 2).

DISCUSSION

The cataracts in these turkeys were bilateral except for 1 bird. The degree of lens involvement varied slightly in the same turkey as well as in different birds. Four of the turkeys were 3 months old or less when the cataracts were first observed. The opaque areas usually increased in size progressively. At no time were there signs of an inflammatory reaction either in the

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Acquired Resistance of Sheep to *Listeria Monocytogenes* and Pilot Studies on Vaccination

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INFORMATION on host resistance to *Listeria monocytogenes* is of interest from the standpoint of further understanding the disease in nature and from the economic aspect of preventive immunization. Data are lacking on the resistance status of ruminant animals following a natural attack of the disease. This lack is due to the nature of listeriosis, in which most of the encephalitis cases are fatal, and because reliable methods for detecting inapparent infections have not yet been described.

The literature on Listeria immunization of animals is in conflict. Graham *et al.*[5] injected bacterin into 8 sheep, but when an intravenous challenge was given, all died. Further, their bacterin did not protect rabbits against an intravenous challenge. In a field trial, Graham and Levine[4] injected broth containing formalin-killed organisms into sheep. The results did not suggest a marked protective effect from the vaccination.

Eveleth *et al.*[2] reported favorable results with bacterins in sheep immunization. Olafson[8] inoculated 20 sheep with live culture, both subcutaneously and intravenously; when the animals were subjected to an intracerebral challenge, 10 survived. In an experiment on blood cell response to *L. monocytogenes*, Olson *et al.*[10] found that 10 of 13 sheep, which had been previously injected by one or more unspecified routes, survived an intracarotid inoculation. Olson *et al.*[9] inoculated live culture subcutaneously during an epizootic in sheep. The attempt at immunization was considered unsuccessful, since losses in the vaccinated and control groups were nearly equal. Osebold and Sawyer[14] showed that mice could acquire resistance when inoculated with sublethal doses of virulent live culture. Hasenclever and Karakawa[6] confirmed these results. Osebold and Sawyer[12] reported that resistance to encephalitis was demonstrated by sheep vaccinated with live virulent organisms.

The present investigation was undertaken to retest these results, to gain information on the persistence of the acquired immunity, and to make preliminary studies on vaccination by means of methods that would be safe from the public health standpoint.

MATERIALS AND METHODS

Culture.—Listeria monocytogenes, strain 3-54, was used for live virulent culture vaccination, for bacterin preparation, and as the challenge culture. The strain, isolated in this laboratory from the brain stem of a sheep, is classified in serologic group 4b.* For maintenance of virulence, the organism was passed serially through mice before each use. Bacterial cells recovered from the mice were in mediums only long enough for the necessary suspensions of organisms to be made before sheep were inoculated.

Immunizing Antigens.—The following antigens were used:

1) Virulent live-cell vaccination was performed by subcutaneous inoculation of strain 3-54, grown 24 hours at 37 C. in brain-heart infusion broth (Difco). Bacterial plate counts were not made for each set of injections, but reference counts indicated that approximately 8×10^8 organisms per ml. could be expected.

2) Bacterin was prepared from Listeria strain 3-54, grown in brain-heart infusion broth at 37 C. for 24 hours. The organisms were killed with 0.3 per cent formalin and adsorbed on an aluminum hydroxide gel. The gel, prepared as described by Marshall and Welker,[7] had a final pH of 7. Killed broth culture was added until the gel was saturated, as determined by microscopic examination of gel-culture mixtures.

Saturated gel was centrifuged 15 minutes at 1,500 r.p.m., the supernatant fluid was discarded, and the gel suspended in an equal volume of 0.85 per cent saline solution containing 1:10,000 thiomersal (Merthiolate).* It was estimated that each

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* Merthiolate is produced by Eli Lilly & Co.,

milliliter of bacterin contained 2.8×10^9 organisms. The vaccine was stored in brown bottles at 4 C. and used 17 days after its preparation.

3) Vaccination with formalin-killed Listeria cells in broth was performed with a brain-heart infusion culture killed with 0.3 per cent formalin. This whole-broth suspension was used in the injections. Bacterial numbers estimated by the plate count method indicated that there were 77×10^7 organisms per milliliter. The suspension was stored in brown glass bottles at 4 C. and used 17 days after its preparation.

4) The commercial bacterin consisted of a washed, formalin-killed culture of Listeria organisms adsorbed on aluminum hydroxide gel.

5) Avirulent live-cell vaccine was a brain-heart infusion broth culture of the Schultz strain. This had been isolated from a human case in California in 1934, and later classified in serologic group 1, by Paterson.[15] The strain, obtained from the American Type Culture Collection, was considered avirulent on the basis of loss of pathogenicity for mice. There was no apparent effect on mice given doses as high as 2.0×10^8 organisms.

Challenge Suspension.—Strain 3-54 was passed serially through mice three times and then seeded to brain-heart infusion broth. After incubation for 24 hours at 37 C., the organisms were washed once in Zobell's solution[16] and suspended in Zobell's solution to one third of the original volume.

Numbers were estimated by the plate-count method, using tryptose agar. The suspension was held 48 hours at 4 C. while the plates were incubated, prior to being counted. The suspension was then used for challenge in sheep, at which time the count was repeated to obviate any error from an unanticipated decrease in numbers during the 48-hour holding period.

Serologic Procedure.—Agglutination tests were performed with somatic antigens prepared from serotype 1 (strain 7973) and serotype 4b (strain 3-54).[13] The organisms were grown in Roux bottles for 48 hours at 37 C. The harvested cell suspensions were held two hours at 100 C., washed three times in 0.85 per cent saline solution, and filtered through gauze. Thiomersal was added to make a final dilution of 1:15,000 in suspensions adjusted to an optical transmission of 80 per cent on a Coleman spectrophotometer at a wave length of 4,500 Å.

Serums were serially diluted in 0.85 per cent saline solution, after which an equal volume of antigen was added. Incubation was carried out at 50 C. for two hours and followed by overnight storage in the refrigerator. Titers were recorded as the serum dilution at which half or more of the antigen was agglutinated.

Bacteriologic Methods.—Bacteriologic isolations from tissues were made on blood agar and blood tellurite agar, and included a periodic reculture examination.[11]

Animals.—The sheep were of mixed breeding and included both ewes and wethers. None of the females was pregnant. The 30 sheep used varied in age from 1 to 6 years. Prechallenge and postchallenge temperatures and agglutinating antibody titers were determined. Animals vaccinated with live cells were kept separate from nonvaccinated controls and animals injected with inactivated organisms. They were brought together at the time of challenge. The intracarotid challenge required minor surgery to expose the carotid artery for a careful inoculation into the vessel.

RESULTS

Experiment 1, Immunization with Virulent Live Organisms.—Two sheep (24 and 105) were given three subcutaneous inoculations of virulent live culture at seven-day intervals; doses were increased from 0.22 ml., 0.44 ml., to 0.66 ml. per kilogram of body weight (table 1). Previous work had indicated that inoculation through this route was not likely to result in encephalitis. Sharp temperature rises followed these injections. The highest reading was 107 F., reached 48 hours after the first dose. The expected antibody response was detected during the vaccination series (graph 1).

The remaining sheep in this phase of the experiment had all been vaccinated and had survived an intracarotid challenge 24 months previously.[12] They entered this study with variable levels of residual agglutinin titers from their previous contact with Listeria antigens. Four sheep (657, 2073, 35, and 28) were challenged to determine the duration of resistance of sheep to *L. monocytogenes* after vaccination with live organisms. Two animals (36 and 598) were given a single recall inoculation of live organisms subcutaneously at the rate of 0.44 ml. per kilogram of body weight.

Thirty days after the last inoculation, these 8 vaccinated animals and 4 nonvaccinated control sheep were challenged by intracarotid inoculation of either 35 or 50 million Listeria cells per kilogram of body weight (table 1).

Marked temperature elevations occurred in both the vaccinated and control animals (graph 2). As reported previously,[12] vaccinated animals regained normal temperatures a day or two after the challenge, whereas the control sheep had fever reactions until near death. The characteristic clinical signs of Listeria encephalitis became manifest in the 4 control animals, with deaths occurring from the fourth through the seventh days. In these acute cases, bacteriologic isolations were made

from the brain and numerous other sites.

One month after challenge, the 8 vaccinated animals were still clinically normal. Blood cultures were all negative. Approximately two months postchallenge, sheep 598 and 657 were observed to be depressed and becoming progressively weaker. They did not demonstrate signs of a central nervous system disorder. Animal 598 died 68 days after challenge, and 657 died two days later. Both animals had interesting lesions of valvular endocarditis. Listeria organisms were readily cultured from the valvular lesions, which were present on both the left atrioventricular and semilunar valves. As would be expected from the continuous showering of organisms into the circulation, numerous tissues, including the brains, yielded positive cultures. Histologic examination of their central nervous systems did not reveal evidence of Listeria encephalitis. The cause of death in both sheep was considered to be cardiac insufficiency.

The remaining 6 vaccinated sheep continued to appear normal, and were killed by electrocution 103 days after challenge. At that time, animals 24 and 105 were subjected to careful bacteriologic examination in a search for persistent foci of infection. Twelve sites in each animal yielded no positive cultures.

The second phase of this experiment included 5 more sheep that were tested to determine the duration of immunity. These animals (34, 100, 323, 614-7, and 2200) had been vaccinated with virulent live cells, and had withstood an intracarotid challenge 27 months previously. Five nonvaccinated control sheep were challenged simultaneously with this group. All 10 of the animals were given 50 million organisms per kilogram of body weight by the intracarotid route (table 1).

Three of the control sheep died, with the usual pattern of high fever until near death, signs of encephalitis, and death four to ten days after challenge. Cultures from brains and other tissues were positive. Two control sheep survived, although 1 (238) had a protracted fever extending through 12 days (graph 2). The 2 surviving controls were killed on the 87th and 136th postchallenge days and carefully examined bacteriologically. Listeria organisms were not recovered.

The temperatures of the 5 vaccinated animals returned to normal a few days after the challenge. The group remained clinically normal throughout the period when nonresistant animals are likely to die

TABLE 1—Resistance of Sheep to Listeria Monocytogenes Following Vaccination with Virulent Live Organisms

Sheep No.	Vaccination	Challenge dose*	No. of days postchallenge until death
CHALLENGE ON MAY 18, 1957			
24	3 subcutaneous	50	..†
105	inoculations	35	..
598	1 subcutaneous recall	50	..
36	inoculation to previously immune sheep	35	..
657	Vaccinated 24 months	50	..
2073	previously	35	..
35		50	..
28		35	..
57	Nonvaccinated	50	7
54	control animals	35	4
160		50	6
159		35	7
CHALLENGE ON SEPTEMBER 21, 1957			
34	Vaccinated 27	50	53
100	months previously	50	..
323		50	..
614-7		50	..
2200		50	..
237	Nonvaccinated	50	..
238	control animals	50	..
239		50	6
240		50	10
241		50	4

* Number indicates how many million organisms per kilogram of body weight were given. † No encephalitis.

TABLE 2—Resistance of Sheep to Listeria Monocytogenes Following Vaccination with Inactivated and Avirulent Organisms

Sheep No.	Vaccination	Challenge dose*	No. of days postchallenge until death
103	2 injections of bacterin	50	9
5137	(formalin killed-aluminum hydroxide adsorbed)	35	6
5125	2 injections of formalin-killed organisms in broth	50	5
5169		35	4
5108	2 injections of commercial bacterin	50	..†
252		35	11
23	3 inoculations of avirulent live organisms**	50	7
104		35	..
57	Nonvaccinated controls	50	7
54		35	4
160		50	6
159		35	7

Challenge inoculations were made May 18, 1957.
* Number indicates how many million organisms per kilogram of body weight were given. † No encephalitis.
** Avirulent for mice.

of encephalitis. However, 50 days after the challenge, sheep 34 suddenly developed encephalitis and died three days later. The organism was recovered from brain, lungs, liver, and spleen. The remaining 4 animals that were challenged to determine their duration of resistance were still alive and healthy one year following the challenge.

Experiment 2, Vaccination with Inactivated and Avirulent Organisms.—Bacterin was injected subcutaneously into 2 sheep (103 and 5137), each weighing 43 kilograms, at a total dose of 40 billion organisms each. The dose was divided into two injections administered 14 days apart (table 2).

Two sheep were injected subcutaneously with formalin-killed Listeria organisms in broth at a dosage comparable to those given bacterin, adjusted according to body weight. Since they were smaller, sheep 5125 was given 26 billion organisms and sheep 5169 was given 39 billion organisms; each dose was divided into two injections given 14 days apart.

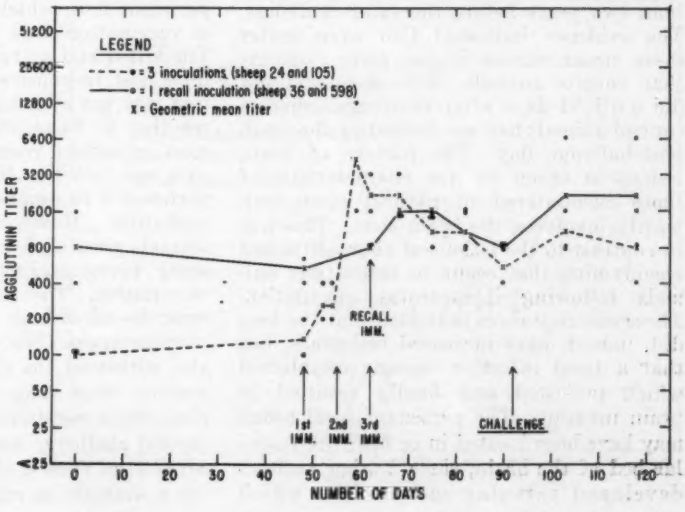
Commercial bacterin was injected subcutaneously into 2 sheep (5108 and 252), each weighing 34 kilograms. Two 5-ml. doses were administered 14 days apart.

A virulent live culture was inoculated subcutaneously into sheep 23 and 104 at seven-day intervals, in three increasing doses (0.08 ml., 0.44 ml., and 0.66 ml. per kilogram of body weight, respectively).

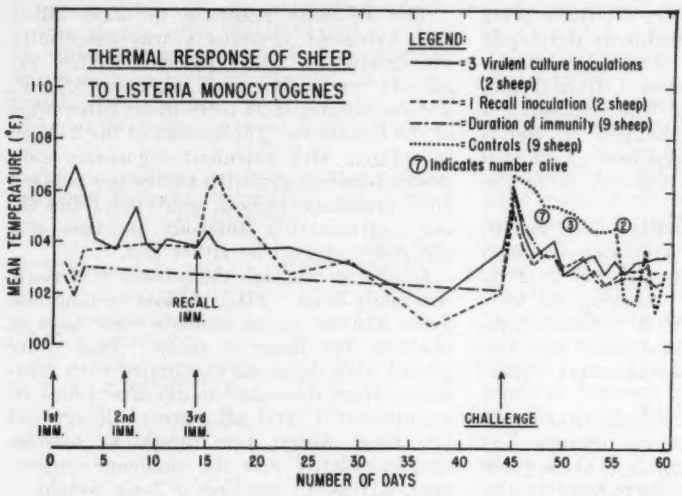
The antibody response to these killed and avirulent organisms was essentially nil (graph 3). Some pyrogenic effect resulted; temperatures as high as 105.8 F. were encountered 24 to 48 hours after some of the injections. The serums of the 2 sheep inoculated with avirulent organisms were tested serologically with antigens prepared from organisms of both serotypes 1 and 4b. An agglutinating antibody increase was not demonstrated by either test.

Challenge through the intracarotid route was made 30 days after the last vaccination. Four nonvaccinated animals were used as controls for these 8 sheep. They were placed with the sheep vaccinated with virulent culture described in the first phase of experiment 1, and all were challenged at one time. Sheep were caught at random and inoculated with the challenge suspension, adjusted according to body weight.

High temperatures, signs of Listeria encephalitis, and death developed in all animals in the vaccinated group. Six of the 8 vaccinated sheep died four to 11 days after challenge. The pattern of response was typical of that encountered in the nonvaccinated control sheep previously described. Listeria organisms were widely distributed in the brains and other tissues cultured. Of the 2 sheep that survived, 1 (5108) had been injected with commercial bacterin and the other (104) was vaccinated with avirulent live organisms. These animals were killed 80 days after



Graph 1—Antibody response in sheep incited by vaccination with virulent Listeria monocytogenes inoculated subcutaneously.



Graph 2—Febrile responses in sheep following the inoculation of virulent Listeria organisms. All animals developed temperature increases from the challenge, but only the control sheep had sustained fevers.

challenge and carefully examined for persistent foci of infection. Twelve tissues from each animal were cultured, but Listeria organisms were not found.

Discussion

Encephalitis in the control sheep and those injected with killed and avirulent Listeria organisms appeared similar in its acute pattern, suggesting a comparable lack of resistance. One encephalitis case occurred in an animal that had been vaccinated with live virulent organisms; however, this sheep (34) was vaccinated more than two years before the fatal challenge. The evidence indicated that even under these circumstances it was more resistant than control animals. This sheep did not die until 53 days after challenge, whereas control animals had succumbed by the tenth postchallenge day. The pattern of brain lesions in sheep 34 was characteristic of those encountered in natural cases, primarily involving the brain stem. This was in contrast to the lesions of choroiditis and ependymitis that occur in susceptible animals following intracarotid inoculation. These circumstances indicated that the host did, indeed, have increased resistance, but that a focal infection became established which persisted and finally resulted in brain invasion. The persistent focal lesion may have been located in or near the vascular bed of the brain, since 2 other animals developed valvular endocarditis which showered the entire blood stream with organisms, but brain invasion did not become established. These two instances of bacterial colonization on heart valves may demonstrate a tendency of the organism to develop foci of infection in the structures of the blood vessel wall. While the endocarditis resulted in death in sheep 657 and 598 after a prolonged period, these animals were resistant to the development of encephalitis.

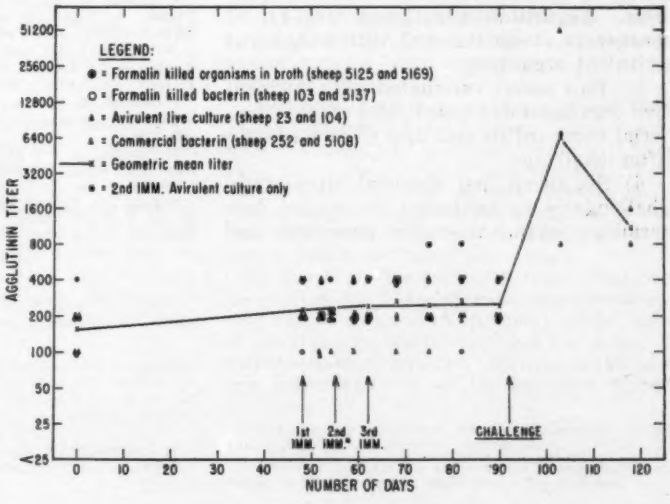
The remarkable persistence of immunity demonstrated in sheep in experiment 1 was in sharp distinction to the outcome of experiment 2, in which more practical means of vaccination were tested on a pilot basis. The killed and avirulent organisms elicited little host response since agglutinin formation was not appreciably stimulated, fever reaction to vaccination was minimal and, most important, resistance to *L. monocytogenes* was lacking. Six of 8 animals in experiment 2 succumbed to acute Listeria encephalitis. However, the survival of 2 animals gives some encouragement for pursuing investigations with these means of vaccination. The survival of the 2 sheep must be viewed in the light of opposing circumstances. Since 2 of 9 control sheep also withstood the challenge, there may be reasons other than the vaccination to explain their resistance. However, the intracarotid challenge was quite severe, and the survival of these 2 sheep may indicate that other animals in experiment 2 might also

have shown a degree of increased resistance in an experiment designed to test response to graded doses of the challenge organism. Further work is needed to clarify this point, since the use of live virulent organisms would not be enthusiastically applied in the field.

Results with virulent live-culture vaccination confirm those previously reported from this laboratory; [12] *i.e.*, that sheep thus vaccinated have a high degree of resistance to Listeria encephalitis as compared to nonvaccinated control animals when subjected to an intracarotid challenge. This method of challenge bypasses the clearance organs, thus assuring that large numbers of organisms will reach the vascular bed of the brain. Since the evidence indicates that the pathogenesis of Listeria encephalitis is hematogenous,[1] this challenge method gives the humoral and cellular elements of the blood an opportunity to participate in the functions of host protection. The experimental disease produced by this procedure differs from natural infections in the distribution and severity of lesions. This difference is probably related to the large inoculum in the experimental procedure and to other undetermined factors. With existing information, intracarotid challenge appears to be the most valid means for evaluating resistance to Listeria encephalitis.

The persistence of antibody formation was determined in the 11 animals used in experiment 1 which had been adequately immunized with Listeria antigens in an investigation completed 24 to 27 months before their use in this study. The geometric mean titer for the group just before challenge was 1:620, which may be compared to the mean titer of 1:36 occurring before any Listeria antigens had been administered. The 9 control animals had a geometric mean titer of 1:86, which is in the range of expected antibody content for so-called normal animals. The origin of these commonly occurring agglutinating antibodies that react with Listeria polysaccharides is still uncertain. It is the opinion of the authors that they are probably not immune antibodies.

The presumption should not be made that higher levels of antibodies in the animals inoculated with live virulent culture indicate that antibodies are solely responsible for the increased resistance. The specific role of antibodies in resistance to listeriosis in ruminants is yet to be determined. It has been shown by Osebold and Sawyer [13] that high-titer sheep serums injected into mice offered no protection against listeriosis in that host. Rather, the presence of antibodies in the sheep inoculated with live virulent organisms may be taken to indicate a host that has been sufficiently affected by the presence of the organism to elaborate antibodies and perhaps activate other important mechanisms of resistance. The possibility of altered cellular resistance, such



Graph 3—Vaccination of sheep by subcutaneous injection of killed and avirulent (for mice) Listeria organisms failed to incite marked antibody response. Notice the high agglutinin titers, stimulated by the challenge inoculation, in the few sheep that survived.

as has been found in tuberculosis by Fong *et al.*,[3] may be a factor.

The killed and avirulent organisms used in this study did not exceed the antigenic threshold. Their apparent lack of immunogenic properties may indicate that resistance to listeriosis will be difficult to achieve unless the agent is alive and capable of surviving in the host for an unknown number of generations. It may then be able to elaborate some necessary substances from its metabolic processes to stimulate the host into a state of altered reactivity. Experiences gained in the past in immunization studies on brucellosis, anthrax, and tuberculosis may be relevant to this question.

SUMMARY

1) Twelve of 13 sheep vaccinated with live virulent Listeria cells one to 27 months before intracarotid challenge were resistant to Listeria encephalitis. The 1 exception, an animal vaccinated 27 months before challenge, died of encephalitis after 53 days.

2) Seven of 9 control sheep died of acute Listeria encephalitis four to ten days after intracarotid challenge.

3) Four to 11 days after intracarotid challenge in 8 sheep, vaccinated one month previously with killed Listeria cells or with live organisms known to be avirulent for mice, 6 of the animals died of acute encephalitis.

4) Antibody response was detected in sheep inoculated with live virulent organisms. Agglutinin production was nil or meager in sheep injected with killed and avirulent organisms.

5) Two sheep vaccinated with virulent live organisms developed fatal cases of bacterial endocarditis and died 68 and 70 days after challenge.

6) Six sheep that survived intracarotid challenge were subjected to careful bacteriologic examination for persistent foci of infection. These studies, made 80 to 136 days after challenge, were uniformly negative.

7) Results are discussed as they relate to host-parasite relationships and prospects for immunizing ruminants.

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Antibiotics for the Control of Vibriosis of Ewes

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VIBRIONIC ABORTION of sheep is an acute infectious disease characterized by abortion during the last half of the gestation period. The incidence of abortion in an infected flock may range from negligible to 70 per cent. The disease is extremely sporadic. It commonly occurs in flocks with no previous history of the disease. It does not often occur in two successive years on the same premises, and may not recur in the same area.

The authors have previously reviewed the literature pertinent to antibiotic treatment of vibriosis in ewes [1,3-9] in a paper [2] which reported on the use of two levels of chlortetracycline (80 and 400 mg. per ewe daily), fed continously, as a preventive of vibrionic abortion. This paper reports another experiment which supplements the results reported in the previous paper.[2]

MATERIALS AND METHODS

Lots 1, 2, and 3 were made up of yearling ewes which were purchased from three sources and brought to the Caldwell Station in the fall of 1957. These yearlings were bred to Panama rams in a field isolated from the premises used for previous vibriosis experiments. The three sources, the number of ewes, and assignment to various lots are as follows:

(a) Eighteen Wyoming whiteface yearlings; 6 were allotted to each of lots 1, 2, and 3. *(b)* Forty Montana whiteface yearlings; 15 were allotted to each of lots 1 and 3, and 10 to lot 2. *(c)* Eleven Idaho Suffolk yearlings; 4 were allotted to each of lots 1 and 3, and 3 to lot 2.

Lot 4 was made up of 26 ewes, 2 years old, which had been used in the previous experiment.[2] In 1957, these ewes had been infected while being continuously fed chlortetracycline; none aborted. Seven had been fed 80 mg. of chlortetracycline per ewe daily and the remaining 18 ewes had been given 400 mg. of the drug daily for the last 60 to 90 days of the gestation period in 1957. All 26 ewes were bred and held separate from the susceptible yearlings until February 15, 1958.

The rams were turned in with both groups of ewes on December 2, 1957, and were removed on January 13, 1958. The ewes were placed in their respective pens on February 15, 1958, and all were fed a pelleted, 20 per cent protein supplement at the rate of 0.5 lb. per ewe daily. The pellets for lot 3 ewes contained sufficient chlortetracycline * to provide 80 mg. of the antibiotic per ewe daily. Pellets were fed daily until all ewes had either lambed or aborted.

TABLE 1—Experimental Design

Lot No.	Inoculum	Status	No. of ewes	Treatment
1	Infected tissues	Yearlings	25	None
2	Infected tissues	Yearlings	19	Penicillin-dihydrostreptomycin injection and chlortetracycline orally 5 days postinoculation
3	Infected tissues	Yearlings	25	Chlortetracycline orally
4	Infected tissues	Fed chlortetracycline and infected but lambed normally in 1957	25	None

On March 14 an inoculum was prepared from *Vibrio fetus*-infected fetal tissues (stomach contents, livers, and fetal membranes) from ewes from eight Idaho flocks affected with vibriosis. These tissues were suspended in saline solution in a Waring blendor. Each ewe was given 2 oz. of this suspension orally, using a dose syringe equipped with a 12-inch ball-tipped dose pipe.

On March 19 (five days after inoculation), the 19 ewes in lot 2 were injected intramuscularly with 4 cc. of a suspension containing 800,000 units of procaine penicillin G. crystalline and 1 Gm. of dihydrostreptomycin sulfate.† Starting on this date and continuing until all the ewes had lambed

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* Supplied by American Cyanamid Company, Pearl River, N.Y., in the form of Aurofac-10.

† Supplied by American Cyanamid Company, Pearl River, N. Y., under the trade name Pro-K-Mycin.

TABLE 2—Results of Administering Certain Antibiotics to Ewes Experimentally Infected with Vibrio Fetus-Infected Tissues

Lot No.	No. pregnant ewes	Lambed	Aborted	Abortion (%)	Vibrio fetus isolated
1	21	6	15	71.0	15
2	16	14	2	12.5	2
3	20	17*	3	15.0	2
4	23	20	3†	13.0	1

* One ewe gave birth at term to a small deformed lamb. Vibrio fetus was not isolated from it.

† One ewe aborted twin lambs seven days after a severe febrile respiratory infection. The fetal membrane was darkened and did not appear typical of vibriosis. Vibrio fetus was not isolated from the lambs.

(June 10), they were fed pellets (0.5 lb. daily) to provide 80 mg. of chlortetracycline per ewe daily. The penicillin and dihydrostreptomycin injection was repeated on March 20.

The ewes in the treated lots were observed closely to see that they were eating the treated feed and for any clinical signs attributable to the drug. Following abortions, no attempt was made to clean or disinfect the premises, and aborting ewes were left in their respective pens. The experimental design is summarized (table 1).

Tissues from all aborted fetuses were cultured on blood agar and in semisolid Albimi brucella broth for *V. fetus*.

Samples of the untreated and treated supplement were collected before and after pelleting for assay for chlortetracycline activity.*

On March 13, before the ewes in lot 3 were started on the treated feed, and at intervals thereafter, blood samples were collected from 5 ewes in lot 1 and 5 ewes in lot 3 for assay for chlortetracycline. These samples were held frozen and submitted for bioassay * by a modified F. D. A. cylinder plate method using *Bacillus cereus* var. *mycoides* No. 9634.

RESULTS

The results of the experiment are summarized (table 2) and the incubation periods for the individual ewes are shown (table 3).

All ewes in lots 2 and 3 appeared to be eating the treated pellets at all times. No difficulty was experienced in starting them on this treated feed. The results of assays of feed for chlortetracycline activity are shown (table 4).

The results of blood assays for chlortetracycline activity are shown (table 5).

DISCUSSION

As reported in previous experiments,[2] the incubation periods in treated ewes which aborted appeared to be longer than those of the untreated controls. *Vibrio fetus* was isolated from vaginal cultures of 5 ewes which gave birth to live, healthy lambs (2 in lot 2, 1 in lot 3, and 2 in lot 4). Cultures were taken in this manner from approximately 20 ewes. This indicates that treatment which inhibits the organism but does not completely eliminate infection may result in an infected ewe giving birth to a normal lamb. Mere prolongation of the incubation period may mean the difference between abortion and apparently normal parturition.

Some loss of chlortetracycline activity (10%) resulted from mixing and pelleting. Although under the conditions of this experiment the loss was not sufficient to affect the outcome of the trial, it should be pointed out that there could be considerable variation in the product from different feed mixing and pelleting plants.

The bioassay method for determining

TABLE 3—Time Interval from Oral Inoculation to Abortion

Lot No.	Postinoculation day																Total No. of abortions
	8	9	11	12	16	18	20	24	27	31	32	35	37	45	50	73	
1	1	1	2	1	2	1	1	1	..	..	1	2	1	1	..	..	15
2	..	..	..	..	1	..	..	..	..	..	..	1	..	..	..	..	2
3	..	..	..	..	..	..	..	1*	1	1	..	..	..	..	..	..	3
4	..	..	..	..	..	..	1	..	..	..	..	..	..	..	1*	1*	3

* Vibrio fetus was not isolated.

TABLE 4—Feed Assays for Chlortetracycline

Sample No.	Feed	Chlortetracycline (mg./lb.)	Chlortetracycline per cent of theoretical
1	Control-mixed	0	.....
2	Control-mixed	0	.....
3	Control-mixed	0	.....
4	Control-pelleted	0	.....
5	Control-pelleted	0	.....
6	Treated-mixed	160	100
7	Treated-mixed	142	89
8	Treated-mixed	112	70
9	Treated-pelleted	160	100
10	Treated-pelleted	144	90
11	Treated-pelleted	149	93

* Bioassay by the American Cyanamid Company, Pearl River, N.Y.

chlortetracycline blood levels is affected by substances (other than the antibiotic) which inhibit the growth of the test organism. The blood of all untreated ewes consumed feeding of 80 mg. of chlortetracycline per ewe from postinoculation day 5 until lambing (2 of 16 pregnant ewes, 12.5%, aborted); and (3) oral inoculation with

TABLE 5—Chlortetracycline Content in Sheep's Blood

Treatment	Sample date	Sheep No. 61	62	63	64	65
Ewes on untreated feed (lot 1)	2–13–58	neg.	neg.	neg.	neg.	neg.
	2–16–58	0.05*	0.07	0.06	0.013	0.02
	2–17–58	neg.	0.02	neg.	neg.	neg.
	2–22–58	0.09	0.05	0.01	0.01	no sample
		86	87	88	89	90
Ewes on treated feed containing 80 mg. of chlortetracycline per ewe daily (lot 3)	2–13–58	0.02	neg.	neg.	neg.	neg.
	2–16–58	0.03	0.03	0.03	neg.	neg.
	2–17–58	0.05	0.03	0.02	neg.	0.01
	2–18–58	0.04	0.01	0.01	neg.	0.03
	2–22–58	0.03	0.02	0.03	neg.	no sample
	3– 1–58	0.04	0.03	0.01	0.01	neg.
	3– 8–58	0.05	0.03	0.02	0.01	neg.
	3–17–58	0.03	0.07	0.02	neg.	0.02
	3–23–58	0.07	0.03	0.04	0.03	0.03
	3–29–58	0.03	0.04	0.02	0.02	0.01
	4– 4–58	0.06	0.05	0.04	0.02	0.01
	4–19–58	0.05	0.05	0.04	0.03	0.02

* Micrograms of chlortetracycline per milliliter of blood.

tained inhibitory substances on two days (Feb. 16 and 22, table 5). This suggests that the inhibitory effect of serum from treated ewes may have been due to substances other than chlortetracycline.

The results leave little doubt that most ewes in lot 4 were immune. However, this does not definitely prove that immunity was developed during the 1956 to 1957 pregnancies while the ewes were being fed chlortetracycline. In the 1957 experiment, the chlortetracycline-containing feed was discontinued immediately after the ewes lambed and, although they were maintained separately from the ewes which aborted, it is possible that the immunity developed after lambing (and removal of the medicated feed) as a result of premise contamination or exposure to an ewe which was shedding the organism after normal lambing.

Summary and Conclusions

Highly significant reductions in the rate of abortion among pregnant ewes, orally infected with *Vibrio fetus*, resulted from (1) feeding 80 mg. of chlortetracycline per ewe daily from 27 days prior to inoculation until they lambed (3 of 20 pregnant ewes, 15.0%, aborted); (2) two daily injections of penicillin and dihydrostreptomycin on postinoculation days 5 and 6 and daily *V. fetus*-infected tissues during the preceding pregnancy while the ewes were being continously fed chlortetracycline (3 of 23 pregnant ewes, 13.0%, aborted). Of the infected pregnant control ewes, 15 of 21 (71.0%) aborted.

These results agree with a previous report that while feeding chlortetracycline continuously at the rate of 80 mg. per ewe daily there will be a highly significant reduction in abortions among ewes infected orally with *V. fetus*. They also indicate that such ewes may become immune even though they do not abort. The data (lot 2) indicate that antibiotic therapy may be of value in a flock infected with vibriosis.

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The Correction of Cobalt or Vitamin B_{12} Deficiency in Cattle by Cobalt Pellet Therapy

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A NEW AND SIMPLE PROCEDURE for correcting cobalt deficiency in sheep was reported in 1958.[5] It was comprised of a heavy pellet * of cobalt oxide which, when compressed and then baked at high temperature, yielded a continuous supply of cobalt in the sheep's rumen. These heavy pellets (commonly called "bullets") were retained for many months, or even years, by virtue of their high specific gravity and were effective in preventing or treating cobalt deficiency. Their efficiency in sheep has been confirmed experimentally in New Zealand,[2] Eire,[11] and Australia.[12] Sheep owners have used them successfully in millions of sheep in cobalt-deficient areas in Australia, New Zealand, Great Britain, and South Africa.

This paper describes a similiar pellet for cattle, and reports on its efficiency in improving the weight gain and raising the liver-vitamin B_{12} concentration in cattle grazing cobalt-deficient pasture.

MATERIALS AND METHODS

The efficiency of cobalt pellets in cattle was determined by comparison of body weights and liver-vitamin B_{12} concentrations of treated and control cattle grazing pastures known or suspected to be deficient in cobalt. The selected cobalt-deficient areas were in South Australia [9] and King Island, Tasmania,[8] on farms where the cattle, facilities, and cooperation of the owners were suitable.

Experimental Cattle.—On one farm, a large herd of Herefords was available (experiments B-9a, B-9b and B-9c). On each of the other farms, the cattle were of mixed ages and breeds, chiefly grade dairy cattle. For each experiment, two groups of cattle were selected on the basis of sex, age, breed, condition, and body weight. The group of cattle to be treated was selected by tossing a coin (except in experiment B-6, described later).

Cattle were weighed on fixed cattle scales or on a mobile scale hauled by a motor vehicle. All the cattle grazed on pasture only. The experiments commenced in winter, when pastures at all locations were green but short. A normal flush of growth occurred in the spring, and pastures were dry when the experiments concluded in the summer.

Copper deficiency is known to occur in grazing sheep and cattle in the cobalt-deficient regions of South Australia and Tasmania where these experiments were located. Before commencing experiments, copper concentrations of liver biopsy samples were determined by established methods.[3] Subnormal to deficient liver-copper concentrations were found at three experimental sites in South Australia (experiments B-9, B-10, and B-12) and one on King Island (experiment B-23). All the cattle in these experiments were therefore treated orally with 100 or 150 ml. of a 5 per cent aqueous solution of copper sulfate at the beginning of the experiment and one or two months later, except that the cattle in experiment B-23 were treated once only.

Cobalt Pellets.—An early experiment had shown that the 5.0-Gm. pellet used for sheep was poorly retained in the bovine paunch. The pellets (fig. 1) used in all these experiments (except experiment B-6) were cylindrical with domed ends, and had the following specifications: cobalt oxide, 90 per cent; weight, 20.0 Gm.; specific gravity, 4.0; length, 2.0 cm.; and diameter, 1.75 cm. They were made by the method described [5] for making 5.0 Gm. pellets for sheep.

In experiment B-6 (the first of this series), the pellets used were similar but weighed only 14.5 Gm.

Administration.—Pellets were administered by means of a plastic hose pipe (1.75-cm. internal diameter and 60 cm. long) that was passed into the esophagus. The pellet was placed in the end of the hose and ejected into the esophagus with a pliable rod. Range cattle were restrained in a chute for treatment.

Liver Biopsy.—Liver samples weighing 500 to 800 mg. were obtained for determination of vitamin B_{12} by aspiration biopsy [10] with the instruments [6,7] devised for liver biopsy of sheep. Surplus blood and moisture were quickly removed on clean dry filter paper (Whatman No. 50). Samples

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* This invention is the subject of patent application in the U.S.A. and other countries.

were immediately transferred to small vials, which were then refrigerated on dry ice in a vacuum flask until they reached the laboratory.

Assay of Vitamin B_{12}.—Liver samples stored at −15 C. showed no significant decrease in vitamin B_{12} content when stored up to six months. Most samples were assayed within one to three weeks, and all within eight weeks after reaching the laboratory.

Retention of Pellets.—The presence of cobalt pellets in the reticulum or the rumen was determined in a few experiments by necropsy, but more often by x-ray fluoroscopy of live animals (fig. 2). This was an adaptation of a method[5] devised for sheep, using a Philips 5-inch image intensifier.†

Four experiments were designed to study the retention of pellets, irrespective of the presence of cobalt deficiency. Additional observations on retention were made by examining pellet-treated cattle in experiments on growth and vitamin B_{12}, or on milk and butter-fat production.

TABLE 1—Mean Body Weights (lb.) of Growing Cattle Treated with 20-Gm. Cobalt Pellets

Exper. No.	Breed	Treatment	No. of animals	Months after treatment							Mean gain per head per day	Mean gain per head
				0	2	4	5	6	7	8		
B-6	Holstein-Friesian yearlings	Control	7	.....	369	.....	.....	488	532	612	1.00	243
		Treated*	7	.....	379	.....	.....	539	612	688	1.28	310
B-9a	Hereford heifers	Control	21	456	480	636	.....	.....	763	.....	1.46	307
		Treated	21	455	500**	656†	.....	.....	797†	.....	1.62	342
B-9c	Hereford steers	Control	10	397	402	565	.....	.....	698	.....	1.43	301
		Treated	9	415	415	605	.....	.....	734	.....	1.51	319
B-20	Jersey steers	Control	5	405	420	527‡	583	.....	.....	.....	1.24	178
		Treated	5	409	477	566	649	.....	.....	.....	1.66	240
B-23	Shorthorn dairy heifers	Control	11	283	277	305	.....	441	.....	.....	0.87	159
		Treated	11	283	279	334**	.....	475§**	.....	.....	1.05	192

* Cattle in experiment B-6 were treated with 14.5-Gm. cobalt pellets. † Significant difference ($P<0.05$). ** Highly significant difference ($P<0.01$). ‡ One control animal showing advanced signs was treated and is included in final mean body weight. § One treated heifer did not gain weight and was slaughtered; it was found to have shed the pellet.

Vitamin B_{12} was extracted from liver samples by an adaptation of a method previously described.[4] Before weighing, liver samples were blotted dry on Whatman No. 50 filter paper. A portion weighing 0.2 to 0.4 Gm. was homogenized with 5 ml. of 0.2 M acetate buffer (pH 4.7) containing 2 mg. of potassium cyanide. Extracts were washed to 20 ml. and autoclaved at 106 C. for ten minutes, filtered, washed with boiling water, adjusted to a pH of 6.8, and made up to suitable volumes for assay. Fresh double-glass-distilled water was used throughout the assay procedure.

The extracts were assayed by using *Lactobacillus leichmannii* 313 and Bacto-B12 assay medium. The preparation of the inoculum and the setting up of the assay tubes was done as described,[4] except that the stock culture of *L. leichmannii* 313 was maintained on stabs of Bacto-Micro assay culture agar. The assay tubes were inoculated with suspensions prepared from six-hour cultures in Bacto-Micro inoculum broth.

Two methods of measuring growth response were compared, *i.e.*, turbidimetrically after 18 hours, and by titration of lactic acid produced after 72 hours of incubation. The methods gave almost identical results, so the more simple turbidimetric method was adopted, using 1 cm. of cells in a Unicam spectrophotometer* at 650 mμ. All concentrations of vitamin B_{12} are expressed as micrograms per gram of wet weight. The water content of liver samples was almost constant and ranged from 69.8 to 73.7 per cent.

RESULTS

Effect of Cobalt Pellets on Body Weight.—Eleven experiments involving 246 cattle on nine different farms were set up to measure the effect of cobalt-pellet treatment on body weight in young cattle. In five of the 11 experiments there was a clear-cut response to treatment (table 1).

In experiments B-9a and B-23, the difference in weight gain in favor of the treated group was highly significant ($P<0.01$).

In each of three experiments (B-6, B-20, and B-23), 1 untreated control showed visible signs of cobalt deficiency during the experiment and required treatment. Each responded quickly to treatment, making gains of 164 lb. in six weeks (steer W45, fig. 3), 70 lb. in five weeks, and 71 lb. in five weeks, respectively.

In experiments B-9a and B-9c, there was no clinical evidence of deficiency in the untreated control cattle. The controls made mean gains of over 300 lb. each during the 210-day experiment, a good growth rate for cattle fed pasture only, but the treated

* Unicam spectrophotometer, manufactured by Unicam Instruments, Cambridge, England.

† Philips image intensifier, manufactured by Philips Electrical Industries, Eindhoven, Holland.

cattle each made additional gains of 35 lb. for heifers and 18 lb. for steers.

In experiment B-6, the first of this series, there were no facilities for weighing cattle at first. The cattle were paired visually and the worst or lightest of each pair was treated with a cobalt pellet, so that the advantage due to treatment in this experiment was actually greater than shown (table 1).

In the remaining six experiments results were equivocal, although the trend was in favor of treatment. When all 11 experiments were considered as replicates of cobalt pellet versus no treatment, the difference in weight gain in favor of treated animals was significant ($P<0.01$–0.02).

Liver Vitamin B_{12} Concentrations.—Liver-vitamin B_{12} concentrations were determined in seven experiments (table 2). Initial levels were low and varied with locality. During the experiments there was generally an increase in liver-vitamin B_{12} in untreated cattle during spring and early summer.

Liver-vitamin B_{12} increased quickly in the treated cattle, and in general reached levels two to three times that of the control cattle at four to seven months after treatment.

Cattle showing signs of cobalt deficiency had liver-vitamin B_{12} concentrations less than 0.10 μg. per gram of wet weight and were as low as 0.02 μg. per gram. The effects of treating such animals with cobalt pellets is shown (fig. 3).

Liver-Vitamin B_{12} Concentration and Weight Gain.—The relationship between the vitamin B_{12} concentration of the liver and corresponding weight gain (expressed as lb./head/day) for individual animals in three experiments is shown (fig. 4). It is best discussed in reference to two periods, *i.e.*, July to November and November to January.

Fig. 1—Cobalt oxide pellets for cattle. Each pellet is 0.75 inch (1.75 cm.) in diameter and 0.80 inch (2 cm.) long with domed ends and weighs 20 Gm.

From July to November the relationship is clear. In all experiments there is a highly significant ($P<0.01$) regression coefficient in the control groups. On the other hand, the regression coefficients for the treated groups are not significant. The point of intersection of the line of regression for control animals meets the line of regression of treated animals at 0.34 in

TABLE 2—Mean Liver-Vitamin B_{12} Concentrations in Growing Cattle Given 20-Gm. Cobalt Pellets

Exper. No.	Location	No. of animals	Months after treatment					
			0	2	4	6	7	8
B-6*	King Island	8 controls	.....	.....	.....	0.15	.....	0.16
		7 treated	.....	.....	.....	0.35	.....	0.32
B-9a	South Australia	10 controls	.....	.....	0.27†	.....	0.41	.....
		10 treated	.....	.....	0.60	.....	0.85	.....
B-10	South Australia	9 controls	0.15	.....	.....	.....	0.38	.....
		9 treated	0.16	.....	.....	.....	1.00	.....
B-12	South Australia	5 controls	0.07	0.14	0.15	.....	0.31	.....
		5 treated	0.08	0.21	0.42	.....	0.80	.....
B-20**	King Island	5 controls	0.03	.....	0.20	0.36	.....	.....
		5 treated	0.03	.....	0.36	0.48	.....	.....
B-22	King Island	3 controls	0.13	.....	0.43	0.56	.....	.....
		7 treated	0.23	.....	0.50	0.73	.....	.....
B-23	King Island	10 controls	0.07‡	.....	0.29	0.25	.....	.....
		10 treated	.....	.....	0.53	0.47	.....	.....

Mean concentrations are given in micrograms per gram of wet weight.
* 14.5-Gm. pellets were used in this experiment. † Six samples only. ** Pastures top dressed with 6 oz. of cobalt sulfate per acre 16 months previously. ‡ Six samples from the 20 animals.

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B-23, 0.36 in B-9a, and 0.20 in B-6, giving a mean value of 0.30 μg. per gram. This value, derived from the data in figure 4, approximates the minimum liver storage of vitamin B_{12} that permits cattle to make optimum weight gains.

TABLE 3—Effect of 20-Gm. Cobalt Pellets on Butterfat Production in Two Dairy Herds

Herd No.	No. of animals	Status	Mean butterfat production (lb.)	
			Previous lactation	Present lactation*
B18	13	Control	250	225
	13	Treated	255	251
B19	14	Control	305	126
	14	Treated	299	163†

* Incomplete. † The difference is significant ($P<0.05$).

This figure agreed quite well with our findings in practice. In experiments where cobalt-treated or control animals had liver-vitamin B_{12} concentrations above 0.30 μg. per gram, there was little difference in weight gains of animals with liver-vitamin B_{12} concentrations between 0.30 and 0.70 μg. per gram. This is illustrated (fig. 4) by comparison of the slopes of the lines of regression for treated and control animals over the July to November period where, in each experiment, the slope of the line for treated animals is not as steep as the slope for the corresponding controls.

During the period of November to January, the relationship between weight gain and liver-vitamin B_{12} concentration was obscure. Presumably, during this period all animals derived sufficient cobalt from the pastures for their metabolic requirements. The control animals made weight gains at the same rate as the treated animals.

The body weight and liver-vitamin B_{12} changes during four experiments are shown (fig. 5). The growth curve of the control groups runs parallel to and below that of the treated groups during this period, particularly in experiments B-6, B-23, and B-9a. However, the treated animals maintained their initial weight advantage and so had over-all better total weight gains for the whole experiment.

Effect of Cobalt Pellets on Milk Production.—Two experiments were commenced in July, 1958, to measure the effect of pellets on milk and butterfat production in herds under official production recording by the Tasmanian Department of Agriculture on King Island.

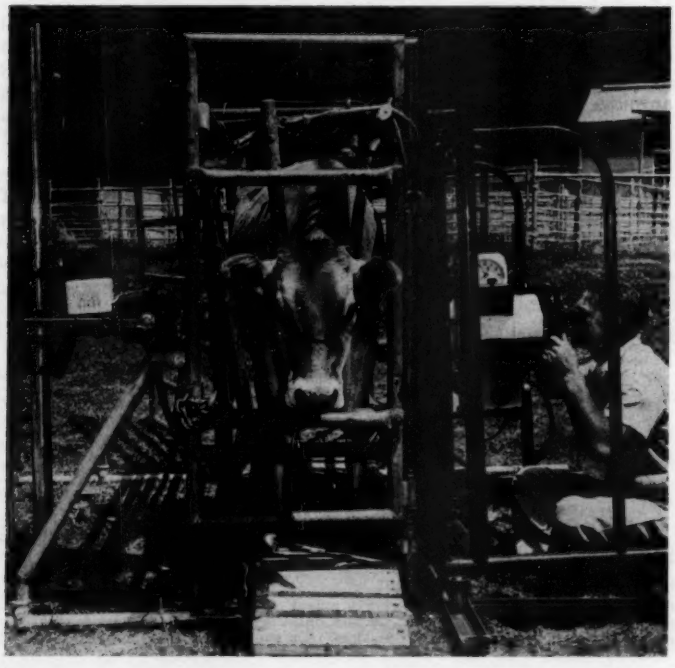

Fig. 2—Fluoroscopy of the rumenoreticulum with the Philips DXI x-ray source and a 5-inch image intensifier. The screen is movable horizontally and vertically. The portable steel chute restrains the animal so that the fluoroscopic screen is 19 to 21 inches from the tube, thereby permitting fluoroscopy at 65 to 85 Kv and 3 to 5 ma.

In each herd, 20 pairs of cows were matched on the basis of breed, age, length of dry period, previous production, and expected date of calving. One cow of each pair was treated with a cobalt pellet in July and again in December. By the end of January, 1959, the number of valid pairs was reduced to 13 and 14 in each herd; pairs that did not calve within 30 days of each other were eliminated.

Some lactations commenced in July, but most in August. The experiments are continuing until the lactation periods are completed. The progress results have not been corrected (e.g., for estrus at the time of recording milk and butterfat production), but are summarized (table 3).

The difference in butterfat production of treated and control cattle reached statistical significance ($P < 0.05$) in experiment B-19, but not in experiment B-18. Both experiments are continuing.

Retention of Pellets.—The retention of 20-Gm. pellets (90% cobalt oxide, specific gravity 4.0) in 281 cattle in 15 experiments is shown (table 4). The presence of pellets was determined at slaughter in two experiments and in all other experiments by x-ray fluoroscopy. Of 210 pellets detected by fluoroscopy in these and other experiments, 197 (94%) were in the reticulum and 13 (6%) were in the rumen.

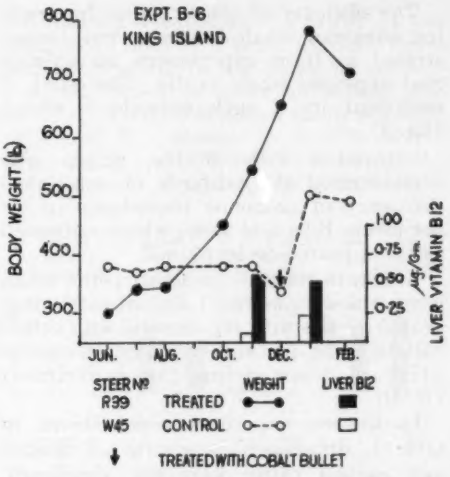

Fig. 3—Body weights and liver-vitamin B_{12} concentrations of a matched pair of steers grazing cobalt-deficient pasture (experiment B-6). Pronounced signs of cobalt deficiency were evident in No. R39 when treated in May and in No. W45 in November. No weighing facilities were available in May.

since in some of the largest animals the pellets may have been present but not detected because of insufficient x-ray penetration. In others, there was sufficient sand in the paunch to obscure a pellet.

TABLE 4—Retention of 20-Gm. Cobalt Pellets in Cattle Determined by x-ray Fluoroscopy or by Slaughter

Class of cattle	No. of experiments*	Months after treatment	No. examined	Pellets retained	
				number	per cent
Under 6 weeks old	3	2–5	18	11	61
6–8 weeks old	2	2–5	19	17	89
6–9 months old	2	3–4	25	24	96
10–20 months old	8	3–7	129	108	84
Lactating dairy cows	5	4–7	90	39	43

* Total of 15 experiments, some including cattle of more than one age group.

In calves treated at less than 6 weeks of age, pellets were poorly retained. Calves treated at 6 weeks of age or older retained pellets satisfactorily for at least two to five months.

In the largest group studied, treated when 10 to 20 months old, 108 of 129 cattle (84%) still retained the pellets when examined four to seven months later.

In lactating dairy cows, only 39 of 90 (43%) still retained the pellets four to seven months later.

Retention rates determined by fluoroscopy are regarded as minimum figures,

Discussion

Adaptation of the sheep pellet for use in cattle required a pellet that would yield sufficient cobalt to meet the daily requirement in cattle and that would be satisfactorily retained in the rumen or reticulum for a reasonable length of time.

The capacity of 20-Gm. cobalt pellets (specific gravity 4.0) to meet the cobalt requirement of cattle is shown by the improved weight gains and liver-vitamin B_{12} concentrations of treated cattle in these experiments (tables 1, 2).

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The efficiency of cobalt pellets for treating advanced cobalt deficiency was demonstrated in three experiments on animals that appeared likely to die. The effect of treatment on 2 such animals is shown (fig. 3).

Treatment with 20-Gm. pellets also demonstrated the existence of subclinical deficiency of economic importance in experiments B-9a and B-9c, where untreated cattle appeared to be normal.

A growth response to cobalt pellet treatment was demonstrated also in cattle that grazed on pastures top dressed with cobalt sulfate (6 oz. per acre) applied 16 months prior to commencing the experiment (B-20).

In the six experiments not shown in table 1, differences in growth of treated and control cattle were not significant. This is attributed to factors such as small numbers of animals in some experiments, uneven distribution of pregnancy in some heifer groups, variable cobalt content of pasture, or the presence of other nutritional limitations, such as insufficient total digestible nutrient or phosphorus intake, affecting the growth of both treated and control cattle. Nevertheless, in all 11 experiments the trend was for cobalt-treated cattle to make better weight gains. When all 11 experiments were examined statistically as replicates of cobalt pellet therapy versus no treatment, the difference in favor of the treated cattle was significant (P between 0.01 and 0.02).

In all experiments where vitamin B_{12} determinations were made, increased growth was associated with increased concentration of vitamin B_{12} in the livers of treated animals (table 2; fig. 5). These results show that cobalt pellets yielded sufficient cobalt for production of adequate vitamin B_{12} for metabolic needs and for stores to accumulate in the liver.

Clinical and growth rate observations of cattle during these experiments suggest that when the liver-vitamin B_{12} concentration is less than 0.10 μg. per gram, cattle do not gain weight and generally show signs of cobalt deficiency, which may be fatal. Cattle having levels between 0.10 and 0.30 μg. per gram may appear healthy and make good weight gains, but may make significantly better gains when treated with cobalt pellets. It is suggested, therefore, that a liver-vitamin B_{12} concentration above 0.30 μg. per gram is necessary for normal weight gain when the cobalt intake from pasture is below the daily requirement and the animal has to draw on liver reserves of vitamin B_{12}.

Similar conclusions were drawn[1] for lambs in New Zealand; *viz.*, cobalt deficiency disease is associated with liver-vitamin B_{12} levels less than 0.10 μg. per gram while healthy lambs have levels above 0.30 μg. per gram.

Liver-vitamin B_{12} determinations were

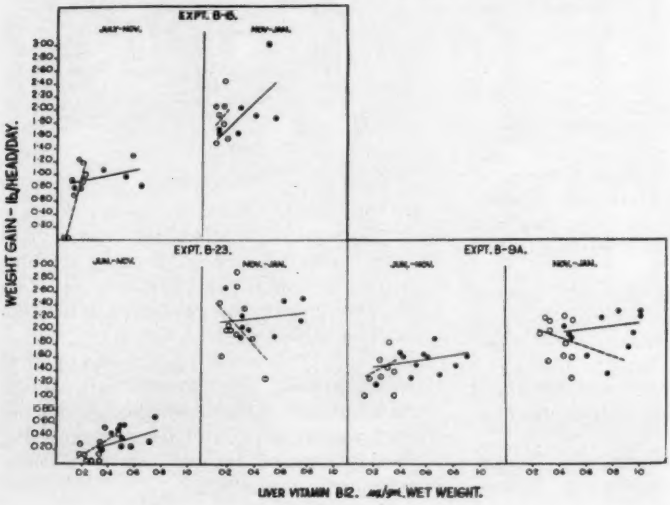

Fig. 4—Relationship of weight gain to final liver-vitamin B_{12} concentrations in individual cattle during two periods in each of three experiments. White circles represent 1 control animal, black circles indicate 1 treated animal; broken line represents line of regression for control groups, and solid line indicates line of regression for treated groups.

made four to seven months after treatment on 6 animals that were found at this time to have shed the pellet. The liver B_{12} concentrations were 0.08, 0.2, 0.43, 0.51, 0.77, and 1.04 μg. per gram. Thus, 4 of these animals apparently retained the pellet long enough to attain an adequate level of liver B_{12}, although the two highest levels might be attributable to the pellet being shed just before fluoroscopy. The two low levels are probably attributable to rejection of the pellet within a few weeks of treatment.

Observations during these experiments confirm the remark that "A striking feature of cobalt deficiency in sheep and cattle is the absence of any signs which can be regarded as specific for this element." [13] The only signs we regularly observed in most experiments was the poorer growth of untreated cattle. Where deficiency was more pronounced, abnormalities observed were weakness and lethargy; pale, rough, erect coat lacking the sheen or bloom of healthy animals; and delayed shedding of the coat in spring and early summer. In a few affected animals there was some ocular discharge, which became crusted around the eyelids and only occasionally flowed onto the face. Diarrhea occurred in a few animals but is apparently an uncommon feature of cobalt deficiency in cattle.

At the commencement of experiment B-6, 1 animal (R39, fig. 3) showed edema of the submaxillary space and, to a lesser extent, of the brisket. This might be considered a sign of combined cobalt and copper deficiency or worm infestation but, as it was corrected promptly by treatment with a cobalt pellet alone, it was attributed to the anemia of cobalt deficiency.

A highly significant response to cobalt supplementation may be obtained in the absence of any signs of deficiency. This occurred in experiment B-9a, where control cattle gained 1.46 lb. per day and treated cattle gained 1.62 lb. per day. At the end of this experiment, the main visible difference in the two groups was the evenness of the coats of treated cattle as compared to those of the control animals, a few of which had rough or pale coats.

Among the several properties of pellets that might influence their retention in the forestomach, it was shown [5] that, in sheep, density is of paramount importance; size and, hence, weight are of lesser importance. Our more limited observations indicate that this applied to cattle also. Further, it was concluded [5] that if a pellet is shed, rejection is by mouth in the course of rumination, and rarely or never by passing posteriorly. We have observed sheep rejecting pellets of low density by mouth during rumination, and conclude that this is the common route of rejection in cattle.

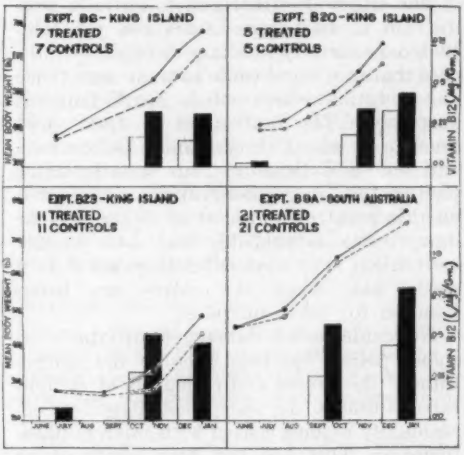

Fig. 5—Mean body weights (solid line, treated; broken line, control animals) and liver-vitamin B_{12} concentrations (black bar, treated; white bar, control animals) of groups of cattle in four experiments.

Calves treated before 6 weeks of age reject many pellets, probably because the rumen and reticulum are not fully developed. Some very young calves rejected them within minutes after the pellet was observed to pass into the thoracic region of the esophagus. Furthermore, in such calves the soft tissues of the pharynx or esophagus may be damaged during treatment; this has occurred in treating very young lambs.

In cattle treated at 2 to 20 months of age, 84 to 96 per cent of pellets administered were present four to seven months later. The fact that the pellets were still effective at this time is shown by the maintenance of high levels of liver-vitamin B_{12} in treated cattle four to eight months after treatment, although the mean values (table 2) include animals shown by fluoroscopy to have lost the pellets and many treated animals not examined by fluoroscopy.

The retention rate in cattle treated at 2 to 20 months of age in these experiments

XUM

is similar to that observed in several hundred sheep we have treated with 5-Gm. cobalt pellets in experiments at several different sites in Victoria.

The lower retention rates in lactating cows are attributed to their greater feed intake and more frequent rumination, which tend to increase the chance of pellets being rejected.

The effect of cobalt pellets on milk production in two experiments not yet completed is already striking enough to indicate that it is worthwhile to treat cows twice per lactation where cobalt insufficiency is suspected. The best times to treat cows seem to be about three months before parturition, and three to four months after parturition; more observations are needed on this point. Treatment of either beef or dairy calves is probably best done at any convenient time soon after they are 6 to 8 weeks old, when the calves are being handled for other purposes.

No evidence of damage attributable to cobalt pellets has been seen in the epithelium of the rumen and reticulum of slaughtered animals. In sheep, cobalt pellets occasionally become coated with calcium phosphate.[5,2] This has not been observed in any of 70 pellets recovered from our experimental cattle.

SUMMARY

Heavy pills or pellets containing 90 per cent cobalt oxide and weighing 5 Gm. have been extensively used for correcting cobalt deficiency in sheep. They are retained in the reticulum or rumen for many months, or even years, by virtue of their specific gravity and weight, and continuously maintain an adequate cobalt concentration in the rumen fluid.

Eleven experiments involving 246 cattle grazing on pasture known or suspected to be deficient in cobalt showed that a single treatment with a similar cobalt pellet weighing 20 Gm. was effective for correcting subclinical or gross cobalt deficiency. Treatment increased the concentration of vitamin B_{12} in the liver and maintained it for at least six to eight months.

Necropsy or fluoroscopic examinations of 281 treated animals showed that calves treated at less than 6 weeks of age did not retain pellets satisfactorily. Of 173 cattle treated at 6 weeks to 20 months of age, 149 retained a pellet for at least two to seven months. Of 90 lactating dairy cows, 39 retained a pellet for four to seven months.

The only sign of cobalt deficiency regularly observed was poorer growth of untreated cattle. Cobalt pellet treatment produced a significant growth response in an experiment in which untreated cattle gained an average of 307 lb. in 210 days.

Clinical cobalt deficiency was associated with liver-vitamin B_{12} concentrations below 0.1 μg. per gram.

The results also indicate that a liver-vitamin B_{12} concentration exceeding 0.3 μg. per gram is necessary to ensure optimum growth when production of the vitamin in the rumen is at a low level and the animal has to draw on its liver reserve to meet daily metabolic requirements.

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XUM

The Relationship of Nasal Discharge to Infection with Infectious Bovine Rhinotracheitis Virus

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INFECTIOUS bovine rhinotracheitis (IBR) is a viral disease of cattle which causes an inflammation of the upper respiratory tract accompanied by a clear nasal discharge, salivation, rapid respiration, anorexia, and fever.[4, 5] Body temperatures vary from 104 to 108 F. and persist within this range for several days to a week. The disease may progress, producing a profuse mucopurulent nasal discharge accompanied by severe dyspnea and, if this occurs, there is often a noticeable weight loss.

In transmission studies, intranasal inoculation with nasal washings from infected animals has been found to be the most satisfactory method for reproducing the disease.[1, 2, 3] However, tracheal exudates, febrile blood, and spleen tissue have also been found satisfactory for reproducing the disease when inoculated intranasally or intravenously,[1] and lung tissue has been found satisfactory for reproducing the disease when inoculated either intranasally or intratracheally.[2] In addition, it has been shown that infectious pustular vulvovaginitis (IPV), a disease of cattle characterized by fever and the formation of pustules on the mucosal surface of the vulva and vagina, is caused by a virus serologically similar to IBR virus. Each virus, when appropriately injected, produces the other disease.[3] Since these would thus appear to be but strains of the same virus, IBR virus may be said to be transmissible by vaginal exudate and by vulvovaginal inoculation.

In the usual feedlot operation, where IBR has been most troublesome, cattle do not usually become infected until they have been on fattening rations 20 to 60 days.[1] The disease may then spread rapidly, infecting all the animals within seven to ten days, or it may spread slowly over a period of 30 to 45 days. In contrast to this relatively slow natural spread, the experimental disease is produced within 24 to 72 hours following inoculation.

Our observations on the relationship of IBR virus to nasal discharge resulted accidentally from an interest in determining if a strain of this virus, which had been transferred through 102 tissue culture passages in primary embryonic bovine kidney tissue, had become modified to the extent that it would no longer produce any signs of infection by either the intranasal or intramuscular route.

MATERIALS AND METHODS

Virus.—The IBR virus used for this work was obtained as the 100th tissue culture passage in primary embryonic bovine kidney tissue.* The virus was passaged twice in our laboratory on the same cells.

The lot of virus used in this experiment was obtained from cells prepared from trypsinized embryonic bovine kidney tissue. The cells were grown in a nutrient medium consisting of Hanks' balanced salt solution, lactalbumin hydrolysate, 10 per cent bovine serum, and antibiotics. This was changed to a mixture of Earle's balanced salt solution and lactalbumin hydrolysate without serum before virus was inoculated. Virus was harvested when cells showed complete cytopathogenic degeneration. The virus was lyophilized to preserve it. The titer of the dried virus on two separate occasions was $10^{-6.9}$ and $10^{-6.6}$ t.c.i.d.$_{50}$ per 0.2 ml. when reconstituted to original volume.

Animals.—The cattle used for this experiment were Hereford steers weighing approximately 600 lb. They had been on full feed for approximately 90 days before the start of this experiment and were normal and in good condition. Serum samples were found to be negative for antibodies against IBR virus. There was no history of IBR ever being present on the premises where they were kept.

Seven steers were inoculated intramuscularly in the neck with 2.0 ml. of virus and 4 were inoculated intranasally with 4.0 ml. of virus diluted to 20 ml. in saline solution. The intranasal inoculation was accomplished by simply inoculating 10 ml. of diluted virus into each nostril. More virus was inoculated intranasally to compensate

From the Department of Biological Research, Norden Laboratories, Lincoln, Neb.

* Obtained from the Veterinary Virus Research Institute, Cornell University, Ithaca, N.Y.

XUM

for possible loss. The atmospheric temperature on the day of inoculation was approximately 0 F. and, since the work was done in an open shed, the syringes and vaccine had to be kept warm. Both groups of animals were kept together before and after inoculation.

Observations.—At the same time each day for 11 days following inoculation, temperatures were taken and the animals were examined for any evidence of infection. At the first sign of nasal discharge in any animal, and for the next two days, nasal swabs were taken from that animal. This was accomplished by swabbing both nostrils with a large cotton swab which was then placed in a screw-topped tube containing 4.5 ml. of Earle's salt solution, lactalbumin hydrolysate, and antibiotics. An attempt was made to collect as much nasal discharge on the swab as possible. The tubes with swabs were frozen within one hour at −40 C.

Virus was detected in the nasal discharge by thawing the tubes, squeezing the swabs thoroughly with a pipette, and then inoculating either 0.2 ml. of the fluid itself or dilutions of it into four or five tissue culture tubes containing 6-day-old cells. All calculations on the amount of virus in the nasal discharge are based on the amount of virus found in the diluting fluid. Since this amounted to perhaps a 1:10 dilution of the original nasal discharge collected, it is possible that because of this dilution factor that very small amounts of virus might have been missed.

Serum Neutralization.—Preinoculation blood samples were obtained three weeks before the start of the experiment and again just before each animal was inoculated. Postinoculation samples were taken at one, two, and three weeks. Blood samples were allowed to clot and the serum was removed and inactivated at 56 F. for 30 minutes. Serum was kept frozen at −20 C. until titrated. Twofold dilutions of serum were made and mixed with approximately 300 t.c.i.d.$_{50}$ of virus. The serum-virus mixture was allowed to incubate for one hour at room temperature before it was inoculated into tissue culture tubes. Four or five tubes were used per dilution. End points were considered to be the dilution showing significant protection at the time the control tube containing 100 to 300 t.c.i.d.$_{50}$ of virus showed complete cytopathogenic destruction.

RESULTS

The daily observations of temperature and the presence or absence of nasal discharge were recorded (table 1). The animals inoculated intramuscularly generally showed an earlier, but not as long lasting, febrile response than those inoculated intranasally. The short incubation time corresponds with that found by others.[2,5] The nasal discharge was intermittent throughout the observation period without particular regard to body temperatures. It was clear, stringy, and mucoid. One week following inoculation, the total feed consumption was down about one third. Steers 201, 203, and 204 stopped eating completely for a day or more and 203 and 204 lost a noticeable amount of weight. Steers 203, 204, and 304 showed definite difficulty in breathing. No diarrhea was noticed. Generally, the animals inoculated by the intranasal route showed a more severe reaction. All recovered without complications and by the time the last blood samples were taken they appeared to be entirely normal.

The results of testing the nasal swabs for virus were tabulated (table 2). The important observation here was that, among the animals with nasal discharge, there was an absolute difference between the two groups. All the animals inoculated intranasally

TABLE 1—Temperatures (F.) and Observations of Nasal Discharge Following Inoculation with Infectious Bovine Rhinotracheitis Virus

Steer (No.)	Pre-inoc.	Postinoculation day 1	2	3	4	5	6	7	8	9	10	11
INTRANASAL INOCULATION												
201	100.2	100.8	104.4	107.4*	105.8*	105.2	105.4*	104.6*	105.4	101.8*	101.4*	100.8*
204	101.4	102.4	101.4	102.8	106.0*	104.0*	105.6*	104.0*	103.4*	101.8	101.8	101.6
301	102.0	102.8	104.8*	105.6*	105.4*	104.4	104.0*	103.8*	104.4*	101.4*	102.0	101.4
304	103.4	102.8	102.2*	105.6	104.8*	104.4	104.4*	102.8*	103.4*	101.2*	101.8*	101.4*
INTRAMUSCULAR INOCULATION												
202	101.0	106.4	105.4*	103.6*	103.6*	102.0	100.8*	101.0*	104.0	101.6	101.4*	102.0*
203	101.4	104.8	104.0	103.8	103.4*	101.2	104.4	103.0*	103.4	102.0	101.6*	101.2
205	103.2	106.0	105.2	104.4	104.4*	103.6	102.0	102.8	103.0*	102.8	102.0	102.4
206	101.0	103.2	104.2	103.6	103.2	102.0	101.8	101.2	102.0*	102.0	101.6	101.0
302	101.2	104.0	104.8*	103.0*	103.4*	101.0	101.0	101.0*	101.4	101.6	101.6	101.0
303	100.8	103.4	104.4	102.8*	105.8*	102.0	100.8*	101.4*	104.0	102.0	102.0	100.4*
305	101.6	105.8	105.0	104.0	103.8	102.0	101.0	101.0	103.4	102.2	100.8	101.0

* Indicates some nasal discharge.

XUM

eliminated virus in their nasal discharge, while those inoculated intramuscularly produced a virus-free nasal discharge. There was no appreciable difference in the appearance or amount of the nasal discharge among the animals showing nasal discharge on any given day. Steer 305 showed no evidence of discharge and steer 206 had a nasal discharge for only one day, which was not checked for virus.

The titer of the virus obtained on consecutive days from the nasal discharges of those animals inoculated intranasally increased above that found on the first day, indicating that virus was produced for a considerable period of time and was not just residual. It is thought that, if any virus multiplication had taken place on the nasal mucosa of those animals inoculated intramuscularly, it would have been found within three days. The virus titers compare favorably with those reported [2] when it is considered that our values, in comparison with reported values, are reduced by at least one log because of the preliminary dilution of the nasal discharge in the collecting tubes.

TABLE 2—Virus Titers Obtained from Nasal Discharge

Route of inoculation	Steer (No.)	Sample 1	Sample 2	Sample 2
Intranasal	201	2.4*	4.5	2.8
	204	2.8	3.8	2.4
	301	1.2	4.3	3.4
	304	NT†	2.8	3.5
Intramuscular	202	....**	....	....
	203	....	....	....
	205			
	302	....	....	....
	303	....	....	....

* Virus titer as log t.c.i.d.$_{50}$/0.2 ml. † Not tested. ** No evidence virus cytopathogenic effect in any tube inoculated with 0.2 ml. of undiluted fluid.

The serum neutralization titers of the animals before inoculation and at one, two, and three weeks following inoculation are shown (table 3). The differences between the two groups are small and thought to be due to the small numbers of animals involved. Since no really significant serological titers were developed until sometime after seven days, it is considered as evidence that the development of specific antibody did not interfere with the release of virus. Intranasal inoculation produced as high an antibody response as intramuscular inoculation.

DISCUSSION

The IBR-IPV virus or complex of viruses is unusual and interesting because it produces two seemingly unrelated clinical entities. The IPV was first reported from Germany in 1888 and has been recognized in dairy cattle in the eastern part of the United States for a long time; IBR was not recognized as a clinical entity until about 1950. The strain known as IPV has been reported to produce fever but no nasal discharge or pustular lesions on the genital organs when inoculated intranasally into cattle.[3] However, virus was apparently produced in the cells of the nasal mucosa, since it was recovered from the nose in amounts larger than those inoculated over a period of days. The other strain of this virus, known as IBR, has been reported to produce fever and pustular lesions on the vulva and vagina but no nasal discharge when inoculated intravaginally.[3] Our experiments have shown that, when a sufficient quantity of IBR virus is inoculated intramuscularly, a fever is produced along with a copious but virus-free nasal discharge. The only consistent reaction observed with the various routes of inoculation was the febrile response.

The fact that a copious nasal discharge without any release of virus was found following intramuscular inoculation indicates that the action of IBR virus upon the nasal mucosa to produce a nasal secretion may be indirect. The fact that not all strains of this virus stimulate a nasal discharge, even when inoculated intranasally, indicates that this may be a distinct charac-

TABLE 3—Serum Neutralization Titers of Steers Inoculated with Infectious Bovine Rhinotracheitis Virus

Steer (No.)	Pre-inoc.	Postinoculation (weeks) 1	2	3
INTRANASAL INOCULATION				
201	.....*	.....	1:32†	1:64
204	.....	.....	1:8	1:64
301	.....	.....	1:8	1:32
304	.....	.....	1:32	1:256
INTRAMUSCULAR INOCULATION				
202	.....	.....	1:4	1:32
203	.....	.....	1:16	1:64
205	.....	1:2	1:8	1:32
206	.....	1:2	1:8	1:32
302	.....	.....	1:4	1:32
303	.....	.....	1:16	1:128
305	.....	.....	1:8	1:64

* Less than 1:2. † Dilution of serum showing significant protection.

XUM

teristic of a virus strain. Reasoning along these lines, it is conceivable that the nasal discharge resulting from other virus infections may not necessarily be a direct result of virus multiplication but some kind of a secondary reaction.

It is unfortunate that, when this experiment was originally planned, the purpose of obtaining nasal swabs was only for the identification of any virus that might be recovered in the event of nasal discharge. Therefore, when nasal discharge continued to be observed beyond the first three days, there seemed to be no reason to change the original plans. It is not unlikely that virus might have been recovered from the nasal passages of those steers inoculated intramuscularly if sampling had been continued. However, despite this drawback, the orginal nasal discharge, at least in those animals inoculated intramuscularly, was not associated with virus multiplication on the cells of the nasal mucosa.

The fact that a larger amount of virus was inoculated than was used in previous experiments reported undoubtedly played an important part in producing this reaction, but this does not explain the mechanism. If this were an allergic reaction and the animals had previously become sensitized to this virus or to any of the ingredients in the tissue culture fluid, it is not known how this might have occurred; there was no previous history, serological or otherwise, of vaccination. Neither IBR or IPV has ever been common in the area from which these steers were obtained. In an attempt to eliminate the possibility of this being an allergic reaction, 1 animal from each group was inoculated intravenously with 100 ml. of undiluted tissue culture fluid, containing the same IBR virus passage, about six weeks after the original inoculation. No reaction other than a one-day rise in temperature in 1 animal was noticed.

The relationship of this finding to those mysterious and elusive viruses believed responsible for shipping fever in cattle and the common cold in people may be pertinent, and it may be that IBR will prove to be a good model to study in an attempt to better understand these other diseases.

SUMMARY

Steers inoculated either intranasally or intramuscularly with a strain of infectious bovine rhinotracheitis virus showed some or all of the clinical signs customarily associated with the disease, including fever, nasal discharge, difficulty in breathing, anorexia, and weight loss. Four steers inoculated intranasally, and 5 of 7 steers inoculated intramuscularly, showed typical copious nasal discharges. The testing of swabs collected from the nostrils of the steers during the first three days following the appearance of nasal discharge showed virus to be absent in the animals inoculated intramuscularly.

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Serum Cholesterol and Paper Electrophoretic Lipoprotein Pattern in Cattle

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SEPARATION of serum lipids by paper electrophoresis and their subsequent identification are of much interest in both the protein-lipid relationship and in the clinical diagnosis of diseases in man.[1,2,4,7] However, the electrophoretic pattern of normal bovine serum lipoproteins has not been studied sufficiently. Rooney,[6] in his work on the paper electrophoretic pattern of bovine serum, studied mainly the protein pattern and suggested further investigation on bovine serum lipoproteins.

For these reasons it was considered worthwhile to analyze the serum lipoprotein pattern of normal cattle, using animals of different ages and both sexes.

MATERIALS AND METHODS

The cattle used in this work were Holstein-Friesians, clinically healthy and free of tuberculosis and brucellosis. They were fed a standard ration of known composition. The 62 animals were allotted to the following five groups: group A, 30 bull calves, 10 to 12 months old; group B, 8 castrated bull calves, 10 to 12 months old; group C, 8 heifers, 10 to 12 months old; group D, 8 cows, seven months' pregnant, 5 years old; and group E, 8 lactating cows, 5 years old.

Food was withheld from the animals for 14 hours before blood samples were taken. Total cholesterol analysis of blood serum was made, using the method of Rappaport and Eichhorn.[5] Paper electrophoretic analysis was carried out in a Grossman-type chamber in 16-hour runs at 110v, using Michaelis buffer (pH 8.6 $\mu \pm 0.1$) and a Whatman No. 1 filter paper served as a carrier. The lipoprotein pattern was established according to the method of Swahn.[8]

RESULTS

Using the paper electrophoretic method, three lipoprotein components were detected. One, which comprised the major part, migrated between albumin and *alpha* globulin; another migrated between albumin and *beta* globulin; and the third remained at its origin. These will be referred to as *alpha*, *beta*, and *gamma* lipoproteins.

Total serum cholesterol and lipoprotein distributions are shown (tables 1, 2). The absolute values (given in mg./ml. of serum) and their standard deviation for total cholesterol as well as for the three paper electrophoretic lipoprotein fractions are shown (table 1). The relative values, in percentage of the lipoprotein distribution of the same groups, are shown (table 2).

The lowest levels of total cholesterol (86.4 mg./100 ml.) occurred in the 10- to 12-month-old bull calves (group A, table 1). However, group C heifers of the same age showed an increase of approximately 33 per cent over the bull calves in group A, with a mean value of 127 mg./100 ml. of serum. This increase is significant at the level of 1 per cent.

The values obtained from the castrated bull calves (group B) ranged between these two groups; the mean value was 100.2 mg./100 ml. of serum. The mean values for total cholesterol obtained from the pregnant cows (group D) and the lactating cows (group E) were the highest values and were practically identical—173.0 and 172.5 mg. per 100 ml. of serum, respectively. In comparison with the animals in the other groups, cows showed a twofold increase over those in groups A and B, and a 60 per cent increase over group C heifers. These results deviate from those reported by Lennon,[3] who found a slightly higher plasma cholesterol content in 3 open heifers than in 3 lactating dairy cows.

The *alpha* lipoprotein fractions showed the same group trends as were shown for total cholesterol values. *Alpha* lipoprotein fractions ranged from 42.0 mg./100 ml. for group A to 130.0 mg./100 ml. for group E. In the *alpha* lipoprotein, as well as in the total cholesterol values (even considering the standard deviation), there was no overlapping of respective values in differ-

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XUM

ent groups, with the exception of the cows in group D and E, where the total cholesterol is identical. The absolute values for *beta* lipoproteins showed very little difference between the groups (17.6–19.8 mg./100 ml.), except for the pregnant cows in group D (23.5 mg./100 ml.). The *gamma* lipoprotein showed the same pattern as the *beta* lipoprotein (23.0 to 24.0 mg./100 ml.) for groups A, B, C, and E, and 30.0 mg./100 ml. for group D.

TABLE 1—Mean Total Cholesterol Values, Lipoprotein Pattern and Standard Deviation

Group	Total cholesterol (mg./100 ml.)	Lipoprotein (mg./100 ml.)		
		Alpha	*Beta*	*Gamma*
A—30 bull calves	86.4 ±11.0	42.0 ±5.9	19.8 ±3.1	24.6 ±2.6
B—8 castrated bull calves	100.2 ±9.6	58.0 ±2.8	19.2 ±2.3	23.0 ±2.5
C—8 heifers	127.0 ±17.2	86.0 ±8.2	18.0 ±3.9	23.0 ±2.5
D—8 pregnant cows	173.0 ±19.0	119.0 ±5.7	23.5 ±5.4	30.0 ±5.2
E—8 lactating cows	172.5 ±11.1	130.0 ±7.6	17.6 ±3.6	24.0 ±5.2

The relative values of the lipoproteins (table 2) obtained by paper electrophoresis showed a constant increase from 48.6 per cent in group A to 75.6 per cent in group E with regard to the *alpha* lipoprotein; both *beta* and *gamma* lipoproteins showed the opposite trend.

The relative values for serum from bull calves were 22.7 per cent *beta* and 28.7 per cent *gamma* lipoproteins. Relative *beta* and *gamma* lipoproteins in the serums of the lactating cows decreased to 10.8 and 13.6 per cent, respectively.

In comparing the recorded values (tables 1, 2), there is a threefold increase in the absolute value of the *alpha* lipoproteins for group A animals over that of cows in group E. At the same time, however, relative values increased only by 65 per cent. Although the relative values for *beta* and *gamma* lipoproteins decreased by more than 50 per cent from group A to group E, the absolute values for these groups remained practically identical.

This would indicate that the increase in total cholesterol is not evenly distributed among all the factions, but acquired by the *alpha* lipoproteins. Consequently, the relative values (expressed in percentages) obtained from paper electrophoresis do not approximate their absolute values.

The gradual increase of the cholesterol and *alpha* lipoproteins from bull calves to lactating cows may be due to the increased secretion of female hormones in the latter groups. The castrated animals, lacking the testosterone secretions, showed the same trend.

STATISTICAL ANALYSIS

A summary of the values from both tables shows that there are considerable differences between the absolute levels for total cholesterol and *alpha* lipoprotein, as well as relative values for *alpha*, *beta*, and *gamma* lipoprotein. Since there were no marked differences in the absolute values of *beta* and *gamma* lipoprotein groups, statistical analysis was limited to fractions showing marked differences. Significance tests were carried out with the following formula:

$$tij = \frac{n_i - n_j}{\sqrt{\frac{6_i^2}{n_i} \quad \frac{6_j^2}{n_j}}}$$

TABLE 2—Paper Electrophoretic Lipoprotein Pattern Expressed as a Percentage of the Total and the Standard Deviation

Group	Lipoprotein distribution (%)		
	Alpha	*Beta*	*Gamma*
A—30 bull calves	48.6 ±6.8	22.7 ±3.6	28.7 ±3.0
B—8 castrated bull calves	57.0 ±2.8	18.8 ±2.3	24.2 ±2.5
C—8 heifers	67.8 ±6.2	14.2 ±3.1	18.0 ±2.1
D—8 pregnant cows	69.0 ±3.3	13.6 ±3.1	17.4 ±3.0
E—8 lactating cows	75.6 ±4.4	10.8 ±2.1	13.6 ±3.0

Total cholesterol values (mg./100 ml.) show statistically significant differences at the level of 1 per cent between groups A and C, C and D, and C and E. Between groups A and B there was only a probable statistical difference at the level of 5 per cent; between groups D and E there was no statistical difference.

The absolute values of *alpha* lipoproteins showed a statistically significant difference at the level of 1 per cent between all groups.

The relative values of *alpha* lipoproteins showed a statistically significant difference at the level of 1 per cent between group A and groups C and D. There was only

a probable statistical difference between groups B and C and groups A and B. There was no statistically significant difference between groups C and D, D and E, and C and E.

The relative values of the *beta* lipoproteins showed a statistically significant difference between groups A and C, A and D, A and E, and B and E.

The relative values of *gamma* lipoproteins for the respective groups showed a pattern of statistically significant differences identical to that of the *beta* lipoproteins.

SUMMARY

Total cholesterol levels and paper electrophoretic lipoprotein patterns were analyzed in the serums of normal cattle of both sexes belonging to different age groups.

Total cholesterol values and *alpha* lipoprotein values for heifers exceeded those of bull calves; castrated bull calves showed intermediate values.

The corresponding total serum cholesterol and *alpha* lipoprotein values of pregnant and lactating cows were significantly higher than those of bull calves, castrated bull calves, and heifers.

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The Inactivation of Foot-and-Mouth Disease Virus, Type A-119, with *Beta*-Propiolactone

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CERTAIN VIRICIDAL characteristics and biologic effects of *beta*-propiolactone (BPL) have been reported [8,10,12] in studies directed toward inactivation of infectious hepatitis virus in blood or plasma without residual toxicity or material change in the constituent proteins. In at least one instance, a combination of ultraviolet light and BPL was necessary to inactivate virus in serum.[15] However, it has been shown that New Jersey type vesicular stomatitis virus contained in 1.0 per cent mouse brain suspension was inactivated when treated with a 0.1 per cent concentration of BPL for four days at 5 to 10 C.[12] In addition, the viruses of lymphocytic choriomeningitis, murine encephalomyelitis, and St. Louis and eastern equine encephalitis contained in 10.0 per cent suspensions of mouse brain were inactivated when exposed to a 0.1 to 0.3 per cent concentration of this chemical at 37 C. for two hours.[7] It has been possible to prepare potent vaccines with BPL-treated equine encephalitis, rabies, and murine encephalomyelitis viruses that are reported to be superior to formalin and phenol-treated vaccines prepared from the same viruses.[11] The inactivation by BPL of vegetative bacteria,[8] fungi,[6] and bacterial spores [3] has also been reported.

It is the purpose of this paper to describe the inactivating effect of BPL on foot-and-mouth disease virus (FMDV).

MATERIALS AND METHODS

Virus.—Foot-and-mouth disease virus, type A, strain 119, was used in these studies in the form of a 10 per cent suspension of infected bovine tongue epithelium. A pool of infected tissue was ground in a mortar, diluted to a 10 per cent suspension with tryptose phosphate broth, and centrifuged in 100-ml. volumes at 2,000 r.p.m. for 20 minutes (900 x g.); the supernatant fluid was used as the starting material.

Preparation of Beta-Propiolactone-Treated Suspensions.—The supernatant fluid from a 10 per cent suspension of infected bovine tongue epithelium was adjusted to a pH of 8.0 with 1 M disodium acid phosphate to effect hydrolysis of the chemical at the reaction temperature of 37 C. The vessels in which the reaction between *beta*-propiolactone and virus took place were screw-capped vials equipped with a bar magnet in the bottom and a thermometer placed in a rubber stopper in the open end.[4] Sufficient amounts of BPL* were measured into each vial to effect 20-ml. volumes of virus suspension in concentrations of 0.5, 0.4, 0.3, 0.2, 0.1, and 0.05 per cent. The virus suspension was added immediately to each vial and mixed with the chemical.

A single vial was prepared containing virus suspension only, and used as a heated virus control. A portion of the supernatant fluid of the original virus suspension served as an untreated virus control. The vials containing BPL plus virus suspension, as well as the vial containing virus only for exposure to heat, were immersed in a water bath over a magnetic stirrer for 15 minutes at a temperature of 37 C. $\pm$ 0.5. After heating, the contents of the individual vials were adjusted to a pH of 7.2 by the addition of 0.05 N HCl where necessary.

Infectivity Tests.—Infectivity tests were performed with tenfold dilutions of the contents of each vial of heated or chemically treated virus, or both, as well as the original virus control. Inoculations were made by the intradermolingual (i.d.l.) route in 2 steers. Grade Hereford steers, 1½ to 2 years old, were inoculated with 0.1 ml. amounts by the i.d.l. route in five sites for each dilution, using four dilutions per tongue and 2 steers per sample.[9] The same dilutions were inoculated into 10 unweaned mice per dilution by the intraperitoneal (i.p.) route. Swiss albino Rockefeller H-strain of mice, obtained from a closed colony maintained by the Plum Island Animal Disease Laboratory, were 5 to 7 days old. Tissue cultures of bovine kidney epithelium were inoculated occasionally with dilutions of the viral preparations to test the infectivity of the treated virus and control virus suspensions.[1]

A second method used to detect infectivity in a treated viral suspension was to inoculate 5.0 ml. of the contents of each vial into each of 2 steers by the i.d.l. route (0.1 ml. per site). In these in-

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TABLE 1—Infectivity Tests in Steers, Mice, and Tissue Cultures of Dilutions of Suspensions of Foot-and-Mouth Disease Virus, Treated with Beta-Propiolactone (BPL) at 37 C.

Virus preparation	Steers* i.d.50/ml.		Steer challenge* l.d.50/ml.		Mice i.d.50/ml.		Tissue cultures i.d.50/ml.
	Exper. 1	Exper. 2	Exper. 1	Exper. 2	Exper. 1	Exper. 2	Exper. 1
Virus control	8.4†	7.4	8.4	7.4	6.7	6.4	8.3
Heated virus control	7.4	6.1	....	....	6.6	5.0	6.3
0.5% BPL**	0.0	....	8.2	....	0.0	....	0.0
0.4% BPL	0.0	....	6.1	....	0.0	....	0.0
0.3% BPL	0.0	0.0	6.6	8.0	0.0	0.0	0.0
0.2% BPL	0.0	2.3	7.2	....	0.0	0.0	0.0
0.1% BPL	....	2.5	....	....	....	0.0	....
0.05% BPL	....	2.0	....	....	....	0.0	....

* Average obtained from 2 steers. † Exponent of the logarithm. ** All BPL and virus heated at 37 C. for 15 minutes.

stances, the presence or absence of FMD was recorded as positive or negative. Other test mediums were not included when this method was employed.

Steers which showed no signs of FMD 14 days after the initial inoculation with inactivated virus were challenged by the inoculation of dilutions of type A-119 virus by the i.d.l. route.[9]

TABLE 2—Infectivity Tests in Steers of 5 ml. of Suspensions of Foot-and-Mouth Disease Virus, Treated with Beta-Propiolactone (BPL) at 37 C.

Virus preparation	Steer infectivity* l.d.50/ml.		Steer challenge* l.d.50/ml.	
	Exper. 3	Exper. 4	Exper. 3	Exper. 4
Virus control	7.7†	7.5	7.7	7.5
Heated virus control	8.0	6.6	....	....
0.4% BPL**	0.0‡	0.0	8.6	6.5
0.3% BPL	0.0	0.0	7.4	7.8
0.2% BPL	+§	0.0	....	8.0

* Average obtained from 2 steers. † Exponent of the logarithm. ** All BPL and virus heated at 37 C. for 15 minutes. ‡ 5-ml. inoculum. § Positive for foot-and-mouth disease.

Complement Fixation.—The BPL-treated, the heated, and the untreated viral preparations were used as antigens in a Traub-Möhlmann complement-fixation test.[10] Serums from the cattle which had been inoculated with the inactive viral preparations, taken at the time of challenge with active virus, were tested for complement-fixing antibody. Appropriate anticomplementary, hemolytic, complement, and hemolytic system controls were performed on the reagents in the test.

Virus Neutralization.—A qualitative test was performed to detect the presence of virus-neutralizing antibody in the serums of cattle inoculated with the BPL-inactivated viral preparations. Two dilutions of each serum (1:10 and 1:100) were mixed in separate tubes with an equal volume of tissue culture-propagated FMDV-A119. Sufficient virus was added to each tube so that each mouse inoculated with the mixture would receive 100 l.d.50 in 0.05 ml. The serum-virus mixtures were incubated at 37 C. for 30 minutes. Each serum-virus mixture was inoculated into 10 suckling mice, 7 to 9 days old. Each mouse was given 0.05 ml. of inoculum by the i.p. route. Known positive antiserum against FMDV and negative serum were mixed with virus, incubated for 30 minutes at 37 C., and inoculated into suckling mice as controls.

RESULTS

Inactivation of Viral Suspensions.—Repeated tests have shown that a minimum concentration of 0.3 per cent BPL was required to inactivate the virus in the supernatant fluid of tissue suspensions as tested by the i.d.l. inoculation of steers with 1 ml. of 10^{-1} to 10^{-4} dilutions (table 1). Similarly treated viral preparations were inoculated by the i.d.l. route in steers in 5-ml. volumes of the 10^{-1} dilution (table 2).

TABLE 3—Complement-Fixing Activity of Foot-and-Mouth Disease Virus in Tissue Suspensions Treated with Beta-Propiolactone (BPL) and Untreated Controls

Antigen preparations	Complement-fixing activity at dilutions of				Antigen control	
	1:10	1:20	1:40	1:80	Anticomplementary	Hemolytic
Virus control	4*	4	4	3	0	4
Heated virus control	4	4	3	0	0	4
0.4% BPL†	4	4	2	0	0	4
0.3% BPL	4	2	2	0	0	4
0.2% BPL	4	4	3	0	0	4
0.1% BPL**	4	4	4	0	0	4
Normal tongue tissue	0	0	0	0	0	4

* 4 = complete fixation or no hemolysis; 0 = complete hemolysis or no fixation. † All BPL and virus heated at 37 C. for 15 minutes. ** Contained living virus.

XUM

Complement Fixation.—The complement-fixing activity of the viral suspensions as antigens was only slightly impaired by treatment with BPL and heat (table 3). No complement-fixing antibodies were found in cattle serums 14 days after inoculation of the treated viral preparations.

Virus Neutralization.—Negative results were obtained in qualitative virus-neutralization tests performed in suckling mice with cattle serum collected 14 days after inoculation by the i.d.l. route with noninfective viral preparations (table 4). The results were negative whether the animals were inoculated with small (1-ml.) volumes of dilutions or larger (5-ml.) volumes of a 10^{-1} dilution of the inactive viral preparations.

TABLE 4—Virus-Neutralization Test in Suckling Mice with Serums Taken from Steers 14 Days after Inoculation with Foot-and-Mouth Disease Virus Antigens Treated with Beta-Propiolactone (BPL)

Preparations for test	Steer inoculum treatment	Dilutions of virus or serums					
		10^{1}	10^{2}	10^{4}	10^{5}	10^{6}	10^{7}
Virus control	.....	.....	.....	10/10*	10/10	2/10	0/10
Pos. serum control	.....	0/10†	0/9	.....	.....	.....	.....
Neg. serum control	.....	10/10	10/10	.....	.....	.....	.....
Serums from steers:							
ET 1340	0.4% BPL	10/10	10/10	.....	.....	.....	.....
ET 1325	0.4% BPL	10/10	10/10	.....	.....	.....	.....
ET 1331	0.3% BPL	10/10	10/10	.....	.....	.....	.....
ET 1299	0.3% BPL	10/10	10/10	.....	.....	.....	.....
ET 1341	0.2% BPL	7/9	10/10	.....	.....	.....	.....
ET 1324	0.2% BPL	9/10	10/10	.....	.....	.....	.....

* Number dead/number inoculated; † each mouse received approximately 100 l.d.$_{50}$ in the serum-virus mixture.

DISCUSSION

The tongue of the steer has been found to be the most sensitive medium for the detection of FMDV, type A-119.[5] However, the tongue is limited by the volume of inoculum which can be retained. Also, the viral content of one lot of infected bovine tongue epithelium may differ from that of another lot.[9] Similarly, the quantity of complement-fixing antigen in one lot of infected tongue epithelium may differ from that of another lot. Neutralizing antibodies in the serum of animals convalescent from FMD or in serums taken after vaccination may be demonstrated.[2] It is difficult to demonstrate complement-fixing antibodies in the serums of steers in the early convalescent stages or after vaccination using ordinary procedures.[13,14] In this study, neither of these antibodies was detected in serums of cattle killed 14 days after inoculation with noninfective viral preparations. These results may be related to the state of the antigen after chemical treatment, to the volume of antigen inoculated, or to the sensitivity of the biologic tests used to detect the presence of the antibodies. In any event, a complement-fixing antigen containing FMDV-A119 in a noninfective state remained after treatment of the infectious suspensions with BPL.

SUMMARY

Beta-propiolactone in a concentration of 0.3 per cent inactivated foot-and-mouth disease virus, type A-119, in the supernatant fluid of suspensions of infected bovine tongue epithelium in 15 minutes at 37 C.

The chemically treated virus suspensions in amounts of 5 ml. of a 10^{-1} dilution did not induce complement-fixing or virus-neutralizing antibodies when inoculated intradermolingually in steers or in the serums tested 14 days later.

The inactivated virus suspensions served as an antigen in a specific complement-fixation test for foot-and-mouth disease virus. Such an antigen displayed no anticomplementary or hemolytic effects.

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XUM

An Evaluation of the Complement-Fixation Test in the Study of Foot-and-Mouth Disease

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THE ENTIRE HISTORY of the use of the complement-fixation test in the study of foot-and-mouth disease has been fraught with controversy. In 1929, Ciuca [5] reported success in the use of the test with antiserums and antigens of guinea pig origin. The report, however, was received with skepticism because of reported failures by other workers in this field. It was not until after the report of Traub and Möhlmann,[19] in 1943, that the complement-fixation test with foot-and-mouth disease virus (FMDV) began to attain some acceptance for the testing of field samples.

In the years following the Traub and Möhlmann report there have been numerous confirmatory publications by independent workers.[1, 4, 10, 15] The most successful and widely accepted test procedure has been the use of known positive hyperimmune guinea pig serums for identification of FMDV antigens of bovine or guinea pig origin. The reverse procedure, employing known positive FMDV antigens to detect antiviral immune bodies in the serums of guinea pigs, has also been widely accepted and utilized.[6] However, the routine detection of complement-fixing antibodies in serums of cattle has been reported unsatisfactory with foot-and-mouth disease (FMD) and vesicular stomatitis viruses.

Frederiks,[7] working with FMDV, reported the successful demonstration of complement-fixing antibodies in bovine serums by eliminating inactivation of the serum at 56 C. for 30 minutes. Serra and Guarini [18] suggested treatment of bovine serums with hypertonic saline solution prior to use in the complement-fixation (CF) procedures, and Nordberg and Schjerning-Thiesen [11, 12] reported success with this method. On the other hand, Brooksby,[1] Eichhorn,[6] Rice and Brooksby,[15] and Rice and McKercher [16] reported failures in the detection of FMDV or vesicular stomatitis virus (VSV) complement-fixing antibodies in bovine serums by any of these methods. Rice and Brooksby [15] and Rice and McKercher,[16] however, did report success when an indirect CF procedure was employed.

Savan, in an unpublished report,[17] described the successful use of a direct 50 per cent end point CF technique for the demonstration of antibodies against VSV in bovine serums. Marucci [8] described the successful application of a direct 50 per cent end point CF technique for detecting antibodies against FMDV in bovine serums, in which a specially prepared antistromal hemolysin was used.

The procedure for the CF test commonly employed at the Plum Island Animal Disease Laboratory has been called the Traub-Möhlmann test which, in brief, consists of washed sheep erythrocytes suspended in saline solution and standardized in a spectrophotometer * at a wave length of 580 mμ to give an optical density reading of 0.500 ± 0.01. Sensitization is accomplished by mixing equal parts of the standard cell suspension and a dilution of commercial hemolysin prepared so that each 0.25 ml. contains 4.0 hemolytic units. Guinea pig complement is prepared so that each 0.25 ml. contains 1.5 units of complement. Each tube in the test contains 0.25 ml. of a given serum dilution, 0.25 ml. of the antigen (a 10% wt./vol. suspension of tissue), and 0.25 ml. of complement (containing 1.5 units). The tubes are shaken and subjected to a 30-minute fixation period at 37 C., then 0.5 ml. of sensitized cells is added and the tubes are again incubated for 30 minutes at 37 C. This test procedure appeared to be fairly satisfactory when guinea pig serums were used; however, when attempts were made

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* Coleman Junior Spectrophotometer, manufactured by Coleman Instruments, Inc., Maywood, Ill.

to study bovine serum samples, the test procedures were found to be inadequate.

The purpose of the present study was to define a simple, yet adequate, direct CF test procedure for detection of antibodies in serum from cattle that had recovered from FMD, and to improve upon the test used with guinea pig serum.

MATERIALS AND METHODS

Diluent.—Isotonic veronal buffered saline solution (VBS) was prepared as described by Mayer *et al.*[9] This contained 0.00015 M of $CaCl_2$ and 0.0005 M of $MgCl_2$, which provided the optimal amounts of Ca^{++} and Mg^{++} ions in the reacting solutions.

Sheep Erythrocytes.—Sheep blood was obtained aseptically, mixed with an equal volume of Alsever's solution, and stored at 4 to 8 C. for at least four days prior to use. Erythrocytes stored in this manner have been found suitable for CF tests for periods as long as eight to ten weeks.[3] When cells were needed for a test, a portion of the blood was removed and washed three times in VBS by centrifugation at 1,800 r.p.m. in an International horizontal-type centrifuge.† After final washing, 2.0 ml. of packed cells was added to 90.0 ml. of VBS, and the suspension standardized in a spectrophotometer ** at a wave length of 580 mμ to give an optical density reading of 0.500 ± 0.01.

Hemolysin.—Stroma obtained by boiling sheep erythrocytes was injected into rabbits, as described by Marucci,[8] and the serums from these rabbits was labeled as "antistromal hemolysin." A standard hemolysin titration was performed, and the optimal dilution of hemolysin selected to sensitize the erythrocyte suspensions as described by Osler *et al.*[10] Sensitization was accomplished by mixing equal parts of the erythrocyte suspension and an optimal dilution of hemolysin and incubating for ten minutes at 37 C. Commercial (Difco) hemolysin at its optimal dilution was also used.

Complement.—Guinea pig serum which had been stored in a dry-ice box was thawed, and dilutions were made. Complement titrations were set up in the presence and the absence of antigen and serum, and 1.5 units of complement (contained in 0.25 ml.) was used in the final test.

Antigens.—Antigens (10% wt./vol. suspension) were prepared from infected and normal tissues which had been stored in a dry-ice box. Block titrations were set up to establish the highest dilution of a given antigen which would give the highest serum titer value, and this was designated as optimal antigen dilution.

Serums.—The bovine serums tested were obtained from animals that had recovered from FMD. Thimerosal (Merthiolate ‡) was added as a preservative in the ratio of 1:10,000, and the serums were stored at 4 to 8 C. The guinea pig serums tested were obtained from animals used in hyperimmunization experiments. The serums were stored at 4 to 8 C. without the addition of preservatives. All serums tested were routinely diluted 1:5 on the day of the test and inactivated at 56 C. for 30 minutes.

Complement-Fixation Test Protocols.—When the direct 50 per cent end point method was used, reagents were placed in each tube in the following order: 0.25 ml. of the appropriate serum dilution, 0.5 ml. of complement containing one and one half 50 per cent hemolytic units, 0.25 ml. of the antigen, and 1.0 ml. of isotonic VBS. The reagents and tubes were held in an ice-water bath during this portion of the test. All tubes were shaken vigorously and placed in a refrigerator overnight at 4 to 8 C. After the overnight fixation period, 0.5 ml. of optimally sensitized cells was added to each tube and the tubes were again shaken vigorously before incubation at 37 C. for 45 minutes. Immediately after the second incubation period, the tubes were placed in multiple carriers and centrifuged at 1,500 r.p.m. for ten minutes. The optical density of the supernatant liquid in each tube was read in the spectrophotometer at a wave length of 541 mμ. A set of control tubes was included with each test, consisting of an antigen, serum, normal or preinoculation serum, and normal-tissue antigen control. In addition, two reference tubes (100 and 0 per cent hemolysis) were included. The percentage of hemolysis in each tube was calculated according to the following relation:

$$\frac{OD^1}{OD^2} \times 100 = H$$

OD^1 is the optical density of the unknown, OD^2 is the optical density of the complete hemolysis control, and H is the percentage hemolysis. The highest dilution showing less than 60 per cent hemolysis was taken as the end point.

In the direct 100 per cent method, the reagents used were 0.25 ml. of the appropriate serum dilution, 0.25 ml. of complement containing one and one half 100 per cent units, and 0.25 ml. of antigen. The tubes were shaken and incubated either at 37 C. for 30 minutes or at 4 to 8 C. for 18 hours. After this fixation period, 0.5 ml. of optimally sensitized cells was added to each tube, and the tubes were again shaken and incubated for 30 minutes at 37 C. The degree of fixation was determined visually, and only those tubes showing 3+ (75%) or 4+ (100%) fixation were regarded as positive.

RESULTS

Attempts were first made to repeat the direct CF test as described by Marucci.[8]

† International Centrifuge, manufactured by International Equipment Co., Boston, Mass.

** Coleman Junior Model 6-B Spectrophotometer, manufactured by Coleman Instruments, Inc., Maywood, Ill.

‡ Produced by Eli Lilly & Co., Indianapolis, Ind.

XUM

After the preliminary steps of producing the rabbit antistromal hemolysin and standardization of reagents, several bovine serum samples were selected for preliminary study. The titers obtained when infected bovine tongue epithelium and infected bovine vesicular fluid were used as antigens are shown (table 1). The control tubes containing normal bovine tongue epithelium as antigen were negative in each case. Although the antibody levels were not high, it was apparent that complement-fixing antibodies could be demonstrated in bovine serums by a direct 50 per cent end point test 14 days postinoculation and that either tongue epithelium or vesicular fluid of bovine origin may be used effectively as an antigen.

TABLE 1—Complement-Fixation Titers of Bovine Serums Using Two Antigens with a Fixation Period of 18 Hours at 4 to 8 C. (50% End Point Method)

Serum No.	Day post-inoculation	Titers of bovine serums	
		Infected bovine tongue epithelium*	Bovine vesicular fluid†
768	0	<10**	<10
	14	40	20
782	0	<10	<10
	14	<10	40
844	0	<10	<10
	14	20	20
785	0	<10	....
	14	<10	....
744	0	<10	....
	14	20	....

* 1:20 suspension, twentieth bovine passage of foot-and-mouth disease virus A-119. † 1:40 suspension twentieth bovine passage of foot-and-mouth disease virus A119. ** Reciprocal of highest serum dilution showing a 50 per cent fixation.

At this stage in the investigation, more than 500 guinea pig serum samples were collected and processed in connection with another project. Each of the serums was to be examined for complement-fixing antibodies in order that a rational hyperimmunization program could be defined. The results of this study are to be reported elsewhere. It became evident that a sensitive, yet simple, CF procedure would facilitate the studies on the guinea pig hyperimmunization project, so efforts were directed toward this end with the hope that the information derived might be extrapolated for use in the bovine serum studies.

A number of guinea pig serums were prepared in serial twofold dilutions and tested against several antigens in parallel series by means of two CF procedures which differed only in period and temperature of fixation. In one procedure, a fixation period of 30 minutes at 37 C. was used; in the other, the fixation period was 18 hours at 4 to 8 C. The two CF procedures were based on the 100 per cent hemolysis end point method, wherein the degree of fixation is interpreted on the basis of macroscopic observation. The results of the two procedures are summarized (table 2).

It was apparent that with guinea pig serums, the use of an 18-hour fixation period at 4 to 8 C. resulted in an eightfold or tenfold increase in demonstrable complement-fixing antibodies with no evidence of nonspecific reactions when normal tissue antigens were used as controls. Additional evidence in support of the specificity of this reaction is presented by the fact that, although the guinea pigs from which the serums were obtained had been inoculated with a guinea pig-adapted strain of virus, equally significant antibody titers were obtained with bovine and guinea pig antigens. Heating the antigens at 56 C. for 30 minutes had no deleterious effects on the CF procedures under investigation.

On the basis of the results obtained with the guinea pig serums, it was deemed advisable to attempt to apply the 100 per cent end point method with the 18-hour fixation period in studies of bovine serums. The results of experiments comparing the 30-minute and 18-hour fixation periods in the direct 100 per cent end point method with bovine serums are summarized (table 3). While complement-fixing antibody was not demonstrated after a 30-minute fixation period, titers as high as 1:80 (and in one instance as high as 1:320) were demonstrated when the fixation period was extended to 18 hours. Positive reactions did not occur with preinoculation serums or with normal-tissue antigens used as controls.

Up to this point, all CF tests were performed employing the erythrocyte hemolytic system sensitized with antistromal hemolysin. An experiment was set up to establish the feasibility of using commercially prepared hemolysin. The data (table 4) indicate that commercially prepared hemolysin resulted in reactions which closely paralleled those obtained with antistromal hemolysin.

XUM

TABLE 2—Comparative Complement-Fixation Titrations of Guinea Pig Serums Using Six Antigens* with Fixation Periods of 30 Minutes at 37 C. and 18 Hours at 4 to 8 C. (100% End Point Method)

Serum pool No.	Day postinoculation	Titers of guinea pig serums					
		Infected guinea pig foot pad epithelium †		Infected bovine tongue epithelium ‡		Normal guinea pig foot pad epithelium	Normal bovine tongue epithelium
		Unheated	Heated **	Unheated	Heated **		
FIXATION PERIOD OF 30 MINUTES AT 37 C.							
35	50	....	....	10§	10	....	....
57	49	....	....	20	20	....	....
61	49	<5	<5	20	10	....	....
62	42	....	....	40	<5	<5	<5
66	42	....	....	40	20	<5	<5
67	49	10	<5	20	10	....	....
71	49	<5	<5	10	10	....	....
73	49	10	<5	20	10	....	....
95	49	<5	<5	20	20	....	....
99	49	<5	<5	40	10	....	....
101	49	<5	<5	20	20	....	....
FIXATION PERIOD OF 18 HOURS AT 4 TO 8 C.							
35	50	....	....	80 §	80	....	....
57	49	....	....	160	160	....	....
61	49	80	80	80	80	....	....
62	42	....	....	160	160	<5	<5
66	42	....	....	320	160	<5	<5
67	49	80	80	80	80	....	....
71	49	80	80	80	80	....	....
73	49	160	160	160	160	....	....
95	49	160	160	160	160	....	....
99	49	160	160	160	160	....	....
101	49	160	160	160	160	....	....

1:40 suspension. † Thirty-first passage of foot-and-mouth disease virus A-119. ** Heated for 30 minutes at 56 C. ‡ Nineteenth passage of foot-and-mouth disease virus A-119. § Reciprocal of the highest serum dilution showing a 3+ or greater degree of fixation.

TABLE 3—Comparative Titrations of Bovine Serums Using Three Antigens with Fixation Periods of 30 Minutes at 37 C. and 18 Hours at 4 to 8 C. (100% End Point Method)

Serum No.	Day post-inoculation	Titers of bovine serums					
		Fixation period of 30 minutes at 37 C.			Fixation period of 18 hours at 4 to 8 C.		
		Infected bovine tongue epithelium	Bovine vesicular fluid	Normal bovine tongue epithelium	Infected bovine tongue epithelium	Bovine vesicular fluid	Normal bovine tongue epithelium
744	13	<10 *	....	<10	80	80	20
745	0	<5	<5	<5	<5	<5	<5
	7	<5	<5	<5	<5	<5	<5
	14	<5	<5	<5	40	80	5
768	14	<10	....	<10	40	....	<10
770	0	<5	<5	<5	<5	<5	<5
	7	<5	<5	<5	<5	10	<5
	14	<5	<5	<5	<5	20	20
782	0	<5	<5	<5	<5	<5	<5
	7	<5	<5	<5	20	40	<5
	13	<5	<5	<5	40	80	<5
784	16	....	....	....	80	80	<10
785	16	....	....	....	40	40	<10
840	0	<5	<5	<5	<5	<5	<5
	5	<5	<5	<5	<5	<5	<5
	9	<5	<5	<5	20	20	<5
	15	<5	<5	<5	<5	20	<5
886	0	....	....	....	10	<5	<5
	14	....	....	....	40	320	5
871	0	<5	<5	<5	<5	10	<5
	14	<5	<5	<5	40	80	<5
	18	<5	<5	<5	40	40	<5
878	0	<5	<5	<5	<5	<5	<5
	7	<5	<5	<5	80	....	10
	14	<5	<5	<5	80	....	<5
886	0	<5	<5	<5	<5	<5	<5
	14	<5	<5	<5	40	160	<5
977	28	....	....	....	40	80	5

* Reciprocal of the highest serum dilution showing a 3+ or greater degree of fixation.

XUM

DISCUSSION

The preliminary nature of the CF tests of bovine serum employing the 50 per cent end point method is, admittedly, rather limited and the antibody titers are low; yet the results indicate that specific antibodies may be demonstrated by this method and thus confirm, at least in part, the more extensive work along this line reported by Marucci.[8] The failure to obtain serum titers as high as those reported by Marucci may be due to the fact that the complement-fixing antigens used were CF tests has long been recognized as frequently enhancing the sensitivity of the test, although it may be accompanied by an increase in the number of nonspecific reactions. The normal-tissue antigens and the preinoculation serums included as controls in the present studies gave no evidence of nonspecific reactions in serum dilutions of 1:10 or greater. Further evidence in support of the specificity of the reactions reported is provided by the fact that the antibody titers were usually as high when the guinea pig serums were

TABLE 4—Comparative Complement-Fixation Titrations of Bovine Serums Using Three Antigens with Antistromal and Commercial Hemolysin with a Fixation Period of 18 Hours at 4 to 8 C. (100% End Point Method)

Serum No.	Day postinoculation	Titers of bovine serums: Infected bovine tongue epithelium	Bovine vesicular fluid	Normal bovine tongue epithelium
ANTISTROMAL HEMOLYSIN				
	0	5 *	....	5
878	7	80	....	10
	14	80	....	5
	0	5	10	5
782	7	40	80	5
	13	40	80	5
COMMERCIAL HEMOLYSIN				
	0	5	....	5
878	7	40	....	5
	14	160	....	5
	0	5	5	5
782	7	20	40	5
	13	40	80	5

* Reciprocal of highest serum dilution showing a 3+ or greater degree of fixation.

from fifth bovine passage tongue epithelial tissue which had been stored for 15 to 18 months in a dry-ice box, or from twentieth passage bovine vesicular fluid which had also been stored in a dry-ice box for more than six months prior to use. It is quite possible that the virus strain used here, A-119, has a different antigenic potential than the A_5 strain used by Marucci. In addition, it was reported by Marucci that peak antibody levels were reached "at two or three weeks postinfection." Serum samples beyond 14 days postinoculation were only rarely available in this study since the cattle were routinely killed at this stage of convalescence.

When the line of investigation was shifted to a study of guinea pig serums and the 100 per cent end point method, the results (table 2) were more pronounced. The importance of the time-temperature relationship during the fixation period of tested with antigens of an heterologous source (bovine) as when tested with antigens of an homologous (guinea pig) source. Brooksby,[2] reporting on a study of guinea pig serums, stated that although the longer fixation period of 18 hours at 4 C. was preferred, it was attended by a loss of specifcity among the O, A, and C types of FMD viral antigen studied. Since the present study was limited by necessity to one strain of virus, no evidence is available on this point, although some increase in the observed cross reactions between types of FMDV may be evident in the report by Marucci [8] and between types of VSV as observed by Savan.[17]

The results obtained with the guinea pig serums led to a re-examination of the bovine serums by the 100 per cent end point method with an 18-hour fixation period. The data (tables 3, 4) indicate that while antibody was not detected with

the 30-minute fixation period, it was frequently detected in dilutions of 1:40 and 1:80 in the same serums when an 18-hour fixation period was used. As was the case with the guinea pig serums, the negative results obtained with the preinoculation serum samples and the normal-tissue antigens provide evidence of the specific nature of the reactions.

Some of the data (table 3) suggest that infective vesicular fluid may be a better antigen than the infective tongue epithelium; however, vesicular fluid is difficult to obtain in large quantities.

Although several investigators have emphasized the value of hemolysin prepared against boiled sheep erythrocyte stroma,[8, 13, 14] work previously done with bovine serum and vsv [17] indicated that the use of hemolysin prepared against whole erythrocytes, obtained commercially, would be satisfactory in the procedures under investigation. The results (table 4), although limited in number, indicate that hemolysin obtained commercially may be satisfactory.

A comparison of the 50 per cent and 100 per cent end point methods, utilizing an 18-hour fixation period, would not be completely valid because the two procedures were not performed at the same time and, therefore, the serum dilution series were not actual replicates. The two procedures differed also in final volumes, number of bovine passages of the antigens, and the amount of complement used.

The two 100 per cent end point methods employed, however, may be compared because they were performed on replicate serum dilutions and the tests differed only in the fixation periods.

SUMMARY

A complement-fixation test, based upon a direct visual 100 per cent end point method with a fixation period of 18 hours at 4 to 8 C., was established as being sensitive enough for demonstration of foot-and-mouth disease antibodies in guinea pig and bovine serums. The method has been reduced to a relatively simple and rapid test which eliminates the time-consuming and tedious steps such as centrifugation of each test tube and examination of the supernatant liquid in the spectrophotometer.

Preliminary experimental data are presented, indicating that commercially available hemolysin prepared against whole erythrocytes may be as satisfactory for use in this test procedure as hemolysin prepared against the boiled erythrocyte stroma.

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XUM

Comparative Experimental Evaluation of Swine Erysipelas Bacterins and Vaccines in Weanling Pigs, with Particular Reference to the Status of Their Dams

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IT HAS BEEN OBSERVED from incomplete studies involving the use of virulent organisms of *Erysipelothrix rhusiopathiae* in conjunction with specific hyperimmune serum that the weanling pigs of immune dams do not respond to vaccination as well as those farrowed by susceptible dams. A partial explanation for this lies in the fact that the offspring of immune dams have a variable degree of passive immunity which persists from several weeks to several months.[8,11] Thus, a state of refractiveness to the development of active immunity by this method of vaccination appeared to have been created by the passive immunity of the weanlings.

When one considers the presence on farms of animals that demonstrated immunity to swine erysipelas by not responding to parenteral exposure to infection, although this disease was not known to be present and vaccination had not been conducted,[3-6,9] it seems logical to assume that a number of the breeding animals would also be immune. Further, their offspring would be passively immune for a variable period of time. Consequently, it would not be unreasonable to assume that on premises where erysipelas is known to be present, the probability of the occurrence of this same situation would be increased.

The object of this experiment was to compare the relative merits of the adsorbate (aluminum hydroxide adsorbed) [10] and lysate (immunegène) [1] bacterins and a vaccine prepared from organisms of reduced virulence (avirulent) [2] in weanling pigs farrowed by dams that were immune or susceptible to erysipelas infection. In addition, a comparison was made of the use of the vaccine alone and the same vaccine in conjunction with specific hyperimmune serum of equine origin in weanling pigs farrowed by susceptible dams.

MATERIALS AND METHODS

Representative samples of commercially produced swine erysipelas biologic agents were obtained through the Animal Inspection and Quarantine Division, ARS, Washington, D.C. A serial lot of aluminum hydroxide adsorbed bacterin was obtained from each of 11 companies; vaccine containing organisms of reduced virulence, from each of three companies; and a serial lot of lysate bacterin, from the producer licensed to make this product in the United States.

In order to select one serial lot of adsorbate bacterin for the experiment, a series of tests was conducted with white mice in conjunction with the government standard serial lot 100-A. A test consisted of making twofold dilutions of the bacterins in physiological saline solution from 1:32 to 1:512, inclusive, injecting subcutaneously 8 mice each with 0.5 ml. per dilution, and exposing the mice 14 days later with 0.1 ml. of a suspension of *Ery. rhusiopathiae* injected subcutaneously. Fourteen days after exposure, an estimation of the potency of each serial lot was arrived at by the method of Reed and Muench.[7] After each of the serial lots of bacterins was tested, the three with the highest potency were retested simultaneously, and the one that gave the highest protective 50 per cent end point was selected for use in this experiment.

The three vaccines prepared from organisms of reduced virulence were reconstituted according to the directions of the manufacturers. Tenfold dilutions were made in chilled peptone water, after which nutrient agar plates were seeded for a bacterial count. White mice, 10 per dilution, were injected subcutaneously with 0.1 ml. of dilutions 10^{-1} through 10^{-4} and exposed 21 days later with 0.1 ml. of a suspension of *Ery. rhusiopathiae*. Selection of the serial lot of vaccine for the experiment was based on the greatest number of survivors 14 days after exposure.

In an effort to be consistent in the exposure for each test, the procedure was as follows: a strain of *Ery. rhusiopathiae* was grown for 24 hours in nutrient broth containing 10 per cent horse serum, distributed equally in vials, and then lyophilized. Preparation of the exposure suspension consisted of reconstitution of the dried cultures with 1.0

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per cent peptone water and of adjustment to 90 per cent transmission (Spectronic 20,* —60° mμ). From this suspension tenfold dilutions were made in chilled peptone water, and then nutrient agar plates were seeded for a bacterial count. The 10^{-4} dilution of the organisms was used in carrying out the exposure. A titration of the exposure culture in mice was conducted with each test. Each dilution series was kept in an ice-water bath until the last mouse was injected.

No preliminary test was made on the serial lot of lysate bacterin because the required test for release of a serial lot of this product is conducted in pigs. We were advised by the producer that the particular serial lot used "had been potency tested."

Twenty-five Landrace-Poland China sows were obtained from the swine nutrition herd of the Animal Husbandry Research Division, Agricultural Research Center, Beltsville, Md., one to six days after being bred to Yorkshire boars. Of the 25 sows, 12 were each immediately vaccinated subcutaneously behind one ear with 1.0 ml. of virulent *Ery. rhusiopathiae* culture and behind the other ear with 20 ml. of specific hyperimmune serum. The site of inoculation of the organisms was examined on the third and sixth days for the presence of a local reaction. Daily temperatures were taken for eight days on all sows. After weaning their litter of pigs, vaccinated and nonvaccinated sows were exposed to infection by the percutaneous, or skin scarification, method on the same day to determine their immunity or susceptibility status. The interval between vaccination and exposure varied from 190 to 210 days.

One of the 12 vaccinated sows failed to conceive; the pigs of one litter all died; and those of another litter were born too late to be of use. Pigs farrowed by the remaining 9 vaccinated dams were weaned at 40 to 63 days of age, a mean of 52 days. Seven of these 9 dams had litters of sufficient numerical size for adequate distribution into three groups (A, B, and C). Each of the seven litters was represented one, two, or three times in each of the three groups and once in a litter control group. Each of the two remaining litters (3 pigs each) was also represented once in the litter control group. Excess pigs from the nine litters were distributed to groups A, B, and C as contact controls. Thus, groups A, B, and C each consisted of a total of 13 principals and 3 contact controls and the litter control group consisted of 9 pigs. The litter controls remained in the same barn, but were separated from the nearest pigs by an empty pen. Group A pigs were each given 5 ml. of the selected serial lot of adsorbate bacterin; group B pigs, 2 ml. of lysate bacterin; and group C pigs, 2 ml. of the selected vaccine containing living organisms of reduced virulence. In every instance, the biologic agent was thoroughly agitated and carefully placed subcutaneously behind the left ear. Daily temperatures were recorded three days before and eight days after vaccination. Observations of the site of vaccination were made on the second through the seventh day, and again at two and four weeks after vaccination. Hemocultures from the principals and contact controls of group C were made on the third, fourth, and fifth days after vaccination by withdrawing aseptically about 20 ml. of blood from the anterior vena cava. The blood was inoculated into 100 ml. of beef infusion broth, which was then incubated for 48 hours at 37 C. Subcultures were examined for the presence of *Ery. rhusiopathiae*.

On the 96th day after vaccination the pigs of groups A, B, and C were exposed to erysipelas infection by the percutaneous method. After the cutaneous reactions were no longer detectable, the animals were placed in outside lots for approximately eight weeks, after which time they were killed and closely examined for abnormalities of the leg joints. Any joint that appeared to deviate from normal was opened and examined bacteriologically.

Of the 13 nonvaccinated sows, 1 failed to conceive and was removed from the experiment. Pigs farrowed by the remaining 12 nonvaccinated dams were weaned at 50 to 60 days of age, a mean of 56 days. Nine litters were of sufficient numerical size for adequate distribution into four groups (A^1, B^1, C^1, and D). Each of the nine litters was represented one or two times in each of the four groups. Of the remaining three litters, one was represented in groups A^1, B^1, and C^1, another was represented in group D, and pigs of the third litter were used as contact controls. All the litters were represented in the litter control group, and excess pigs were distributed to the test groups to serve as contact controls. Thus, groups A^1, B^1, C^1, and D each consisted of a total of 13 principals and 3 contact controls. Vaccination and observations of groups A^1, B^1, and C^1 were identical to groups A, B, and C. Pigs of group D were given 2 ml. of the vaccine of reduced virulence and 5 ml. of commercial hyperimmune serum, placed subcutaneously behind the left and right ears, respectively. The serum was comparable in potency to the previous standard B.A.I. lot 7. Hemocultures were made also from groups C^1 and D. Exposure to erysipelas infection of this series of pigs was made in the same manner and time interval after vaccination as groups A, B, and C. A final examination of the pigs was made approximately 12 weeks after being placed in the outside lots.

All of the pigs were kept in pens with concrete floors and runs and were fed a balanced ration which contained an antibiotic (chlortetracycline) at the rate of 6 lb. per ton of feed. After the dams had farrowed, a mixture of iron and copper sulfate was applied to the mammary glands, and reduced iron was given to the baby pigs per os shortly after birth and before weaning.

Demonstration of either immunity or susceptibility was based on the response to the percutaneous method of exposure. Four lyophilized strains

* Manufactured by Bausch and Lomb, Rochester, N.Y.

of *Ery. rhusiopathiae*, indentified as S-192, HC-585, 422-1, and NF-4, were used for exposure of swine in the experiment. The exposure strains were reconstituted in nutrient broth and incubated for 24 hours at 37 C. prior to use. These cultures were always placed in the same sequence on one side of a pig. Daily observations were made after the exposure until the cutaneous reactions were not detectable. Temperatures were recorded daily until they registered below 103 F. Classification of the results of scarification was as follows: (1) completely immune (I)—pigs showing no local reactions; (2) incompletely immune (II)—pigs with local reactions which did not persist or become extensive, yet could not be considered negative; (3) susceptible, local infection (SL)—pigs with cutaneous reactions on one or more scarification lines which persisted and became extensive but remained localized; and (4) susceptible, general infection (SG)—pigs that developed general infections. General infection was characterized by one or more individual cutaneous or urticarious lesions appearing on the side opposite the one that was scarified.

RESULTS

When tested simultaneously, the three adsorbate bacterins with the highest initial protective 50 per cent end points and the standard gave 50 per cent protection in calculated dilutions of 1:180, 1:159, 1:105, and 1:100, respectively. On the basis of these results in mice, the bacterin giving a 50 per cent end point of 1:180 was used in this experiment.

Of the mice injected with vaccines of reduced virulence (X, Y, and Z), 1 mouse died in each of the groups injected with 10^{-1} dilutions of vaccines X and Z, whereas none died or were ill in the groups injected with either the undiluted or higher dilutions of vaccine during the 14-day period after exposure. Consequently, the death of 2 mice was not considered significant. No deaths occurred in mice injected with vaccine Y. Since this test did not indicate any significant difference in the immunizing potential of these vaccines, one was arbitrarily selected for use in the experiment. Viability counts of three vials of the selected vaccine gave an average of 57.8×10^7 cells per milliliter of reconstituted vaccine.

Susceptibility Status of Dams.—After vaccination of the 12 bred sows with virulent cultures and immune serum, 7 showed a local cutaneous reaction at the site of culture injection. The cutaneous reaction was similar to an individual cutaneous or an urticarious lesion associated with a general swine erysipelas infection. Since the reaction involved the soft tissue behind the ear, the edges did not present such a clearly defined geometric outline as was seen on the other areas of the skin. By extension of the borders of the local reaction, an area of erythema and variable swelling covering 2 by 2½ to 4 by 6 inches was observed by the sixth day after vaccination. On black-pigmented skin, the local reaction and its area of extension could be easily palpated. Of the remaining 5 sows, 4 presented no indication of a local reaction and 1 developed a small (½ x ½ in.) purple area which never increased in size and, consequently, was not considered a sign of local infection. There was a slight but definite increase in the postvaccination temperature of sows that developed a local cutaneous reaction. Although the reacting sows would not be considered to have demonstrated severe systemic response, 3 manifested temperature peaks of 104.2, 104.4, and 104.6 F. on the day after vaccination. In addition, 1 of these sows showed a second peak of 104.0 F. on the fifth day. However, at no time was there an apparent deviation in either the appetite or general attitude.

Of the 12 vaccinated sows, 11 were classified, after percutaneous exposure, as CI and 1 as SL. Although the sow was classified as susceptible, she would still be judged as demonstrating some degree of residual immunity against swine erysipelas because she showed only a cutaneous reaction to one of the four exposure strains and no apparent systemic response. Because this sow was classified as susceptible, her pigs were not considered in the presentation of the results.

Of the 12 nonvaccinated sows, 8 were classified as I and 4 as susceptible. Three of the 4 susceptible animals had local reactions and were classified as SL and 1 was classified as SG. All 4 susceptible sows reacted to the four exposure strains and demonstrated a definite systemic response.

One of the vaccinated immune dams died approximately six weeks after experimental exposure. The cause of death could not be ascertained, and *Ery. rhusiopathiae* was not recovered from either the body organs or seven leg joints. A final examination of the 23 remaining sows, eight weeks after experimental exposure, disclosed no visible abnormality of the leg joints.

Reaction to Vaccination in Weanling Pigs.—A nodule, approximately ¾ by 1

inch in size, developed in the weanling pigs at the site of vaccination with adsorbate and lysate bacterins. The nodules were firm and, in most instances, well circumscribed. Usually a slight erythema of the skin could be detected, which gradually disappeared by the seventh day after vaccination. Several of the nodules formed by the lysate bacterin developed a small area of purplish-red discoloration with a yellowish center. Two weeks after vaccination, little change could be seen in nodules produced by the adsorbate bacterin, while those of the lysate bacterin definitely had become smaller. After four weeks, the adsorbate nodules, although reduced in size, could be easily palpated; the lysate nodules either had disappeared or were present in the form of a small indurated area. No reaction at any time could be detected at the site of vaccination of weanling pigs injected with the vaccine containing living organisms of reduced virulence.

There was no significant systemic response after vaccination with any of the three biologic agents alone or the one used in combination with serum, as judged on the basis of daily observations and temperatures following vaccination. Hemocultures of weanling pigs injected with the vaccine of reduced virulence, either alone or with serum, and of their respective contact controls did not yield *Ery. rhusiopathiae.*

Protection Induced in Pigs.—To present the results of weanling pig vaccination, it was necessary to realign the pigs according to the status of the dams. This was necessary because of the 12 nonvaccinated dams, 4 were susceptible to percutaneous exposure (NV-S), whereas the other 8 were completely immune (NV-I). Since this condition prevailed in the nonvaccinated dams, it was considered probable that a similar condition existed in the other sows when they were vaccinated. Additional evidence, although not conclusive, was that 4 of the vaccinated sows showed a local reaction at the site of vaccination and 8 did not. The former sows were identified as being susceptible when vaccinated and immune to percutaneous exposure (SV-I) and the latter sows as being immune at the time of vaccination and of percutaneous exposure (IV-I).

Realignment of the pigs under the four conditions of the dam status retained the uniformity of the vaccinated groups. The only imbalance occurred between the num-

TABLE 1—The Effect of the Status of Dams on Response of Their Weanling Pigs to the Same Biologic Agent

Group	Status of dams	Biologic agent	No. of pigs	Response						
				Cutaneous			Temperature (F.)				
				I	SL	SG	Below 103	103 or above	104 or above	105 or above	106 or above
A	SV-I	Adsorbate	5	0	5	0	0	5	2	1	0
A	IV-I	Adsorbate	6	1	4	1	0	6	4	2	1
A[1]	NV-I	Adsorbate	7	1	6	0	3	4	2	1	1
A[1]	NV-S	Adsorbate	6	0	6	0	1	5	1	1	1
B	SV-I	Lysate	6	0	6	0	0	6	1	1	0
B	IV-I	Lysate	6	0	4	2	0	6	4	2	1
B[1]	NV-I	Lysate	7	0	3	4	0	7	6	4	0
B[1]	NV-S	Lysate	6	0	0	6	0	6	5	3	2
C	SV-I	Vaccine*	6	0	6	0	0	6	2	1	0
C	IV-I	Vaccine*	6	0	5	1	0	6	4	1	0
C[1]	NV-I	Vaccine*	7	0	4	3	0	7	6	2	1
C[1]	NV-S	Vaccine*	6	0	3	3	0	6	3	2	1
D	NV-I	Vaccine* and serum	7	0	5	2	0	7	7	5	3
D	NV-S	Vaccine* and serum	5	0	2	3	0	5	5	4	3
Control	SV-I	.....	8	0	5	3	0	8	8	4	1
Control	IV-I	.....	8	0	4	4	0	8	8	6	0
Control	NV-I	.....	17	0	11	6	0	17	17	14	2
Control	NV-S	.....	6	0	0	6	0	6	5	3	3

I = completely immune; SL = susceptible, local infection; SG = susceptible, general infection; SV-I = susceptible at time of vaccination and immune to percutaneous exposure; IV-I = immune at time of vaccination and immune to percutaneous exposure; NV-I = not vaccinated; immune to percutaneous exposure; NV-S = not vaccinated; susceptible to percutaneous exposure.

* Vaccine contained living organisms of reduced virulence (avirulent).

ber of contact controls and the number of litter controls representing the NV-S and NV-I dams for the vaccinated groups A^1, B^1, C^1, and D. This realignment did not involve a change in the physical conduct of the experiment as outlined, although the number of pigs in the various groups were reduced below a desirable level.

When exposed to percutaneous infection 96 days after vaccination, the vaccinated pigs were classified as susceptible with the exception of 2 immune pigs that had been given the adsorbate bacterin. The contact and litter control pigs were all classified as susceptible. Although practically all the vaccinated pigs were classified as susceptible, the percutaneous results and temperature responses presented differences which were interpreted as reflections of degrees of susceptibility.

The effect of the status of dams on the response of their weanling pigs to the same biologic agent is tabulated (table 1).

Weanling pigs that were given adsorbate bacterin did not show any particular difference in the cutaneous response to percutaneous exposure, regardless of dam's status. However, the somewhat lower temperature response of the offspring of the nonvaccinated dams, particularly of those of the NV-I dams, suggested they were less susceptible than the offspring of the vaccinated dams.

Weanling pigs given lysate bacterin showed a difference in both cutaneous and temperature responses which were related to the status of their dams. These responses indicated a trend of increasing susceptibility which is expressed in the following sequence by the origin of the pigs: SV-I, IV-I, NV-I, and NV-S dams. The most notable difference was that more pigs (10 of 13) farrowed by nonvaccinated dams showed general infection and a corresponding temperature response than those (2 of 12) farrowed by vaccinated dams; however, this difference was due mainly to the more divergent position of the offspring of the SV-I (6 SL-0 SG) and NV-S dams (0 SL-6 SG).

Weanling pigs given the vaccine of reduced virulence without serum showed a difference in their response which could be related to the vaccination status of their dams. More pigs (6 of 13) from nonvaccinated dams showed general infection and a corresponding temperature response than those (1 of 12) from vaccinated dams. There was no significant difference between

TABLE 2—The Response of Weanling Pigs of Dams of Similar Status to Different Biologic Agents

Group	Status of dams	Biologic agent	No. of pigs	Response							
				Cutaneous			Temperature (F.)				
				I	SL	SG	Below 103	103 or above	104 or above	105 or above	106 or above
A	SV-I	Adsorbate	5	0	5	0	0	5	2	1	0
B	SV-I	Lysate	6	0	6	0	0	6	1	1	0
C	SV-I	Vaccine*	6	0	6	0	0	6	2	1	0
Control	SV-I	.....	8	0	5	3	0	8	8	4	1
A	IV-I	Adsorbate	6	1	4	1	0	6	4	2	1
B	IV-I	Lysate	6	0	4	2	0	6	4	2	1
C	IV-I	Vaccine*	6	0	5	1	0	6	4	1	0
Control	IV-I	.....	8	0	4	4	0	8	8	6	0
A^1	NV-I	Adsorbate	7	1	6	0	3	4	2	1	1
B^1	NV-I	Lysate	7	0	3	4	0	7	6	4	0
C^1	NV-I	Vaccine*	7	0	4	3	0	7	6	2	2
D	NV-I	Vaccine* plus serum	7	0	5	2	0	7	7	5	3
Control	NV-I	.....	17	0	11	6	0	17	17	14	2
A^1	NV-S	Adsorbate	6	0	6	0	1	5	1	1	1
B^1	NV-S	Lysate	6	0	0	6	0	6	5	3	2
C^1	NV-S	Vaccine*	6	0	3	3	0	6	3	2	1
D	NV-S	Vaccine* plus serum	5	0	2	3	0	5	5	4	3
Control	NV-S	.....	6	0	0	6	0	6	5	3	3

I = completely immune; SL = susceptible, local infection; SG = susceptible, general infection; SV-I = susceptible at time of vaccination and immune to percutaneous exposure; IV-I = immune at time of vaccination and immune to percutaneous exposure; NV-I = not vaccinated; immune to percutaneous exposure; NV-S = not vaccinated; susceptible to percutaneous exposure.

* Vaccine contained living organisms of reduced virulence (avirulent).

the pigs from NV-I and those from NV-S dams.

Weanling pigs that were given serum in addition to the vaccine of reduced virulence showed a difference in the cutaneous response. A greater proportion of pigs (3 of 5) from NV-S dams showed evidence of general infection than pigs (2 of 7) from NV-I dams; this conclusion was supported by the temperature response of 106 F. or above.

The nonvaccinated contact control and litter control pigs farrowed by NV-S dams appeared to be more susceptible than the controls from other types of dams; all of these pigs showed evidence of general infection. Although this evidence of difference in susceptibility was not substantiated by the temperature response of 105 F. or above, it was supported by that of 106 F. or above.

The response of weanling pigs of dams of similar status to different biologic agents is presented (table 2).

Pigs farrowed by SV-I dams and vaccinated with adsorbate bacterin, lysate bacterin, or the vaccine of reduced virulence showed no apparent differences in the cutaneous and temperature responses after exposure to infection. The corresponding controls were more susceptible than the vaccinated pigs.

Vaccinated pigs that had been farrowed by IV-I dams showed, with the exception of 1 pig, no significant differences in the cutaneous and temperature responses after exposure to infection. The exception was a pig given adsorbate bacterin which was completely immune to percutaneous exposure. The corresponding controls were more susceptible than the vaccinated pigs.

Vaccinated pigs that had been farrowed by NV-I dams showed differences in degree of susceptibility, as evidenced by the cutaneous and temperature response to exposure. Those given adsorbate bacterin demonstrated less susceptibility than the other three groups of vaccinated pigs. On the basis of the cutaneous responses there was a trend of increasing group susceptibility in the following order: ((a) pigs given the vaccine of reduced virulence in combination with serum, (b) the vaccine without serum, and (c) the lysate bacterin. However, this trend was reversed in the temperature responses of these three groups of pigs at 106 F. or over. On the basis of the cutaneous and temperature responses, the corresponding controls were more susceptible than the pigs given adsorbate bacterin, whereas in susceptibility they appeared similiar to the other three groups of vaccinated pigs.

Vaccinated pigs that had been farrowed by NV-S dams showed differences in degree of susceptibility, as evidenced by the cutaneous and temperature responses to exposure. Those given the adsorbate bacterin again demonstrated less susceptibility than the other three groups of vaccinated pigs. Although no significant differences could be seen in the cutaneous responses, the higher temperature responses of the pigs injected with the vaccine of reduced virulence in combination with serum suggested that the pigs were more susceptible than those injected with the vaccine without serum. On the basis of the cutaneous responses, the corresponding controls appeared to be more susceptible than pigs given adsorbate bacterin or the vaccine of reduced virulence alone or in combination with the serum, whereas the controls were equal in susceptibility to those given the lysate bacterin. With the exception of the pigs given adsorbate bacterin and of those given the vaccine without serum, the temperature responses of the other groups of pigs were similar to those of the controls.

At the termination of the experiment, 3 pigs showed abnormalities of the leg joints. Two pigs had small subcutaneous abscesses which did not involve the joint capsule; the third pig, although showing no obvious disturbance of locomotion, had an enlarged hock which contained an excessive amount of serosanguineous synovial fluid and periarticular connective tissue proliferation. A pure culture of *Ery. rhusiopathiae* was isolated from this joint only. This pig had originated from a SV-I dam and had been vaccinated with adsorbate bacterin.

DISCUSSION

The presence of naturally immunized sows in this group of nonvaccinated mature animals established that swine erysipelas existed in low-grade form in the herd from which the bred sows were obtained. This was disconcerting because all except 2 nonvaccinated sows from the herd used prior to 1957 were found to be susceptible, and a number of them died after percutaneous

exposure to erysipelas infection. The two exceptions occurred in 1953.

Although the dams that did not show a local cutaneous reaction at the site of vaccination were considered to be immune at that time, it was possible that 1 or more of them could have been susceptible. This opinion is based on a previous study involving vaccination of swine with virulent culture and serum. Some of those that showed a local cutaneous reaction at the site of inoculation were subsequently found to be immune, whereas others were susceptible. Furthermore, there were a few that did not show a local reaction and were later found to be immune. This variability of dam status may account for the intermediate response of the offspring of IV-I dams when compared with the response of those of SV-I and NV-I dams. Although this situation could not be seen in the groups that were given adsorbate bacterin, it was apparent in the groups that were given the lysate bacterin or the vaccine of reduced virulence (table 1). The immunizing effect of these two biologic agents appeared to be enhanced in weanling pigs from immune dams. However, the degree of enhancement was the least in the offspring of NV-I dams, most in those from SV-I dams, and intermediate in those from IV-I dams (table 2). No explanation is offered to account for this apparent enhancement effect; however, some of the effect must have been due to the presence of different levels of residual passive immunity in the offspring of immune dams when exposed percutaneously to infection. This supposition is supported by the response of the nonvaccinated control pigs from the same four groups of dams, in that the offspring of the NV-S dams appeared more susceptible than those of the vaccinated and nonvaccinated immune dams.

When the cutaneous reactions to the four exposure strains were reviewed, the pigs given the adsorbate bacterin were judged to be less susceptible than indicated in the results (table 1). Of 22 pigs classified as susceptible, 11 demonstrated either complete or incomplete immunity to three of the four exposure strains. Furthermore, this kind of response was not confined to pigs in any one of the four classifications of dam origin. Only 2 of 25 susceptible pigs that had been given the lysate bacterin showed this kind of response, and it did not occur in any pigs that had been given the vaccine of reduced virulence either alone or with serum, or in the nonvaccinated controls.

This experiment did not adequately demonstrate what effect, if any, the immune serum had on the immunizing potential of the vaccine of reduced virulence. However, in the limited number of offspring from only the NV-S dams, which eliminated the factor of passive immunity, the pigs given serum in conjunction with the vaccine were more susceptible than those given vaccine alone. For this reason, it is not impossible that immune serum could have an inhibiting effect on the vaccine of reduced virulence when administered simultaneously.

SUMMARY

Weanling pigs were vaccinated against swine erysipelas with aluminum hydroxide adsorbed bacterin, lysate (immunegène) bacterin, or vaccine prepared from living organisms of reduced virulence (avirulent) alone, or the latter vaccine in conjunction with specific hyperimmune serum. The pigs were offspring of vaccinated and nonvaccinated dams and there exposed to erysipelas infection 96 days after vaccination by the percutaneous (skin scarification) method.

Except for 2 pigs that had been given adsorbate bacterin, all were found to be susceptible, including the nonvaccinated contact and litter control pigs.

Although the majority of weanling pigs were classified as susceptible when exposed after vaccination, the results of the percutaneous exposure demonstrated some differences between vaccinated groups which were interpreted as variations in the degree of susceptibility. These differences also were considered to be associated with the biologic agent they had been given, and the status of the dams.

The essential points derived from the results of this experiment are as follows:

1) Pigs given adsorbate bacterin appeared to be similar in susceptibility, regardless of the vaccination or susceptibility status of their dams.

2) Pigs given lysate bacterin were less susceptible when derived from vaccinated and from nonvaccinated immune dams than from nonvaccinated susceptible dams.

3) Pigs given vaccine of reduced virulence without serum were less susceptible

when derived from vaccinated immune dams than from nonvaccinated dams that were either susceptible or immune.

4) Although differences were observed in the response of pigs given vaccine of reduced virulence alone and pigs given this vaccine in combination with hyperimmune serum, they could not be considered significant.

5) Pigs given adsorbate bacterin, lysate bacterin or the vaccine of reduced virulence were similar in susceptibility only when they were derived from vaccinated immune dams.

6) The nonvaccinated control pigs from vaccinated and from nonvaccinated immune dams were similar in susceptibility, but were less susceptible than those from nonvaccinated susceptible dams.

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Action of Certain Viruses, *Mycoplasma Hyorhinis*, and Nasal Trichomonads on Swine Tissue Cultures

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TISSUE CULTURE systems offer valuable techniques for the study of swine viruses. Information on swine tissue culture systems is contained in the work of Beran *et al.*,[2] Madin *et al.*,[11] Piercy and Ferris,[14] Gustafson and Pomerat,[5] Hancock *et al.*,[6] Hopkins,[8] Young *et al.*,[19] Ichihara,[9] Lee *et al.*,[10] Bankowski and Pfeifer,[1] Emery and York,[4] and Schwarz *et al.*[16]

The purpose of this paper is to report the development of serial passage swine nasal mucosa, swine lung, swine kidney, and swine endothelium cell cultures and, in addition, to report the techniques for growth of primary cell cultures of young swine kidney and swine lung. The effects of hog cholera virus, pseudorabies virus, swine influenza virus, Newcastle disease virus, eastern equine encephalomyelitis virus, western equine encephalomyelitis virus, virus pneumonia of pigs, *Mycoplasma hyorhinis*, and swine nasal trichomonads on these cell cultures are reported.

MATERIALS AND METHODS

Glassware Preparation.—Pyrex, Kimax, Neutraglass, or Duraglass glassware was used. Because of undesirable leakage of CO_2, silicone rubber stoppers employed in a portion of this work were discarded in favor of cafe-au-lait rubber stoppers.* All glassware and stainless steel equipment was cleaned with sodium metasilicate pentahydrate † solution by the process suggested by Scherer[15] except that distilled water was used so that no water-softening agent was needed. The final rinse water was prepared by passage through a mixed-bed ion exchange column. This cleaning process has been utilized for two and one half years with no apparent residue accumulation. After being wrapped in aluminum foil, all glass and metal items were sterilized at 160 C. dry heat for two hours. Rubber stoppers, tubing, and necessary assembled items were sterilized in an autoclave at 15 lb. of pressure for 15 minutes.

Preparation of Mediums.—Distilled water used for mediums was prepared by passage through a mixed-bed ion exchange, and had a conductivity equivalent of approximately 0 parts per million NaCl. Three balanced salt solutions were used. The first was the modified Earle's balanced salt solution described by Madin *et al.*[11] This balanced salt solution with 0.5 per cent lactalbumin enzymatic hydrolysate added is designated as M(LA). The second balanced salt solution was that suggested by Marcus *et al.*,[12] and is designated as BSA. The third solution, designated as E(LA), was that suggested by Earle *et al.*,[3] prepared with 0.2 Gm. of $MgSO_4$ per liter and enriched with 0.5 per cent lactalbumin enzymatic hydrolysate. These solutions were prepared from reagent grade chemicals.

Cattle serum (CS) from choice grade 800- to 1,000-lb. animals, swine serum (SS) from aged boars, horse serum (HS) from a single donor, and lamb serum (LS) from single and groups of 2 to 4 market lambs were utilized. The clotted blood was transferred to a close-mesh stainless steel basket suspended over a container. The serums were clarified by two centrifugations at 2,000 r.p.m. for 15 minutes and then heated at 56 C. for 30 minutes for inactivation. Storage was at −20 C. At this temperature, the serums were satisfactory for use at least one year after collection. Chicken embryo extract (EE_{50}) was prepared by the procedure of Waymouth and White.[18] Penicillin and streptomycin were routinely incorporated in the complete medium at levels of 200 units and 100 μg. per milliliter. Difco 1:250 trypsin, Difco yeastolate, and 100x Eagles * enrichments were employed.

All complete mediums used for the serial passage cell lines were sterilized by nitrogen atmosphere pressure filtration through Selas No. 02 filters. After sterilization at 10 lb. of pressure for ten minutes, the mediums used for the primary swine kidney and lung cultures were prepared by combining a 7.5 per cent $NaHCO_3$ solution with a balanced salt solution enriched with 0.5 per cent enzymatic lactalbumin hydrolysate. Filter-sterilized serum and sterile antibiotic solution were added.

Tissue Source.—The swine tissue culture systems utilized tissue from fetal swine, young swine, or market-age swine. In areas removed from central markets, tissues from fetal swine or market-age swine were difficult to secure or expensive when found. Therefore, the tissue culture systems

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* Manufactured by A. Daigger and Co., Chicago, Ill.

† Produced by Metsogranular Philadelphia Quartz Co., Philadelphia, Pa.

* 100x Eagles (Eg), manufactured by Microbiological Associates, Washington, D.C.

developed in this work utilized swine tissues derived from 1-day-old to 8-week-old pigs from a disease-controlled herd maintained at Iowa State University. This herd has been free of any detectable evidence of respiratory disease since its inception over four years ago.

The pigs were stunned with electricity and exsanguinated. The kidney was aseptically freed from its capsule and a generous portion of the renal pelvis removed. No attempt was made to collect cortex only. Lung tissue was aseptically collected from the margins of the lung so that only the smaller bronchi wereincluded. The mucosa was stripped from the posterior half of the nasal utrbinate which is relatively bacteria-free. The nasal mucosa tissue strips were rinsed in several changes of BSA containing penicillin and streptomycin. Swine endothelium cell line was initiated from cells scraped from the lining of the portal vein after several rinses with BSA. These cells were scraped free with a sterile scalpel and were not subjected to trypsin digestion. All tissue except endothelium was minced with sterile stainless steel scissors into 2- to 3-mm. fragments and washed three to five times with BSA. The cells used to initiate serial passage cell lines were harvested from pooled material from several cycles of digestion at 37 C. for 15 minutes in BSA containing 0.33 per cent trypsin.

The primary swine kidney and lung cultures were initiated from cell suspensions obtained by the continuous-flow digestion system suggested by Melnick,[13] utilizing 0.33 per cent trypsin in BSA at room temperature. It was possible to reduce the three to four hours required to digest the lung tissue to one to two hours by overnight treatment with trypsin at 5 C. A small gauze bag was fashioned over the discharge tube of the continuous-flow digestion flask to filter the cells as they were collected. The cell suspensions were centrifuged at 1,000 r.p.m. for five minutes, the supernatant fluid discarded, and the cells resuspended in 10 to 20 volumes of growth medium. This suspension was centrifuged at 600 r.p.m. for three minutes and the supernatant fluid discarded. This process was repeated. The cells were then suspended in growth medium at a 1:300 dilution. The kidney cells had 65 to 80 per cent viability and the swine lung cells 85 to 90 per cent viability, as determined by the eosin Y strain exclusion method of Hanks and Wallace.[7] The cell suspensions were dispensed in 1.0-ml. amounts in 13- by 100-mm. tubes, in 1.5-ml. amounts in 16- by 150-mm. tubes, and in 12.0-ml. amounts in 8-oz. prescription bottles.

Cover slip preparations of the cell sheet were prepared by inclusion of an 11- by 22-mm. cover slip in the 16- by 150-mm. tubes. The volume of cell suspension was increased to 2 ml. When stained cell sheet preparations were desired, the cover slips with adherent cell sheets were removed from the tubes, rinsed for a few seconds in 0.85 per cent NaCl solution, and transferred to Bouin's fixative plus 5 per cent acetic acid. The cover slips remained in this fixative for 30 minutes, after which they were transferred into two changes of 70 per cent ethyl alcohol for a total of 20 minutes. The cover slip preparations were then placed in 1:50 Giemsa's stain for four to 24 hours at 37 C. The cover slip preparations were destained to the desired intensity by immersion for a few seconds in 70 per cent ethyl alcohol and then quickly passed through 95 per cent and absolute ethyl alcohol. This was followed by immersion in two changes of xylol for a total of ten minutes and mounting.

The growth medium used for the serial passage swine kidney, lung, endothelium, and nasal mucosa cell lines was $M(LA)_{\infty}8S_{50}EE50_{2}$. After growth had occurred, the cell sheet was liberated by incubation with BSA containing 0.04 per cent trypsin and 0.25 per cent disodium dihydrogen ethylenediaminetetraacetate dihydrate† at 37 C. for six to eight minutes. The cells liberated from one culture were used to initiate three comparable cultures.

The growth medium used for the primary swine kidney cell cultures was $M(LA)_{\infty}CS_{20}$. The maintenance medium was $E(LA)_{\infty}CS_{10}$. The primary swine lung cultures were grown in $M(LA)_{\infty}CS_{5}Eg$ and maintained in $E(LA)$ + 0.05 per cent yeastolate. All cultures remained stationary at 37 C. except the primary swine lung culture, which was maintained at 37 C. in roller drums.

RESULTS

It has been possible to secure serial passage of swine kidney through 105 transfers, swine nasal mucosa through 87 transfers, swine lung through 65 transfers, and swine endothelium through 23 transfers. In adaptation of these cell lines to serial passage, the first few transfers grew readily, but from about the fourth to the tenth transfers an additional two or three days' time was required to form cell sheets. After this period of adjustment, there was relatively rapid cell growth with the formation of cell sheets in two to four days.

The serial passage swine kidney cells and the primary swine kidney cells were both epithelial in appearance (fig. 1, 7). The serial passage swine lung cells were predominantly epithelial, while the primary swine lung cells were usually a mixture of fibroblasts and epithelium (fig. 2, 3). The serial passage nasal mucosa remained a mixture of epithelial-type cell islands surrounded by cells suggestive of mesoderm (fig. 4). The swine endothelium cell culture appeared to be fibroblasts after a few transfers (fig. 5), even though a distinctive cobblestone appearance was observed dur-

† Produced by TitraVer-Hach Chemical Co., Ames, Iowa.

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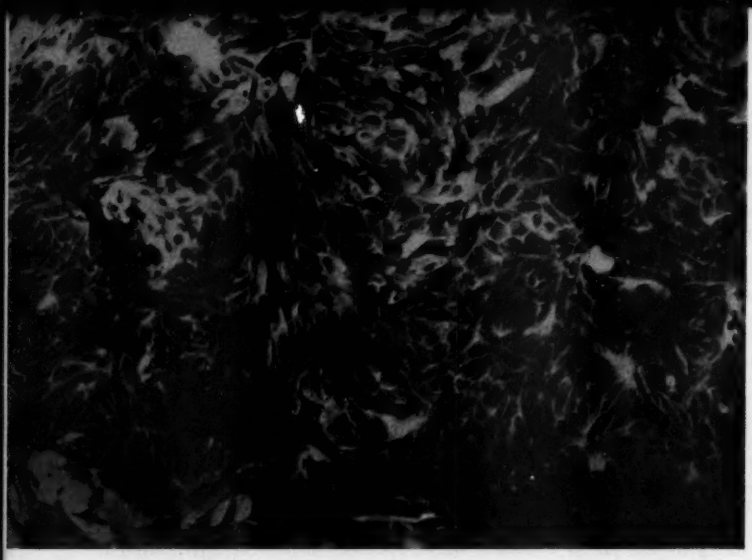

Fig. 1—Primary swine kidney cell culture 4 days old. Giemsa's stain; x 100.

ing the first two transfers. Earle's balanced salt solution was substituted for M(LA) in order to maintain the serial passage cell sheets for about five days. After this time, the cell sheets degenerated in spite of frequent medium changes.

The primary swine lung culture cell sheets could be maintained for seven to ten days by utilization of E(LA)+0.05 per cent yeastolate. The incorporation of cattle, swine, lamb, or horse serum in the maintenance medium hastened destruction of the cell sheet. An unusual tendency of the nucleus to elongate and curve until it formed a "C" shape was observed in some of the primary swine lung cultures. In some cases, the two ends of the nucleus joined and formed doughnut-shaped structures.

The primary swine kidney cultures were more granular than the serial passage cell line cultures. The substitution of lamb or horse serum for cattle serum in the growth medium produced less granular cells, but the cell sheets were not as complete and could not be maintained as long as when cattle serum was used. Maintenance medium was changed every five days and the cell sheets remained usable for three to four weeks.

After the 23rd serial passage, the swine endothelium cell line was discontinued due to contamination with kidney cells (fig. 6). The swine kidney cell overgrowth proggressed until the fibroblastic cells typical of the endothelium cell line were in the minority.

The susceptibility of these cell lines to

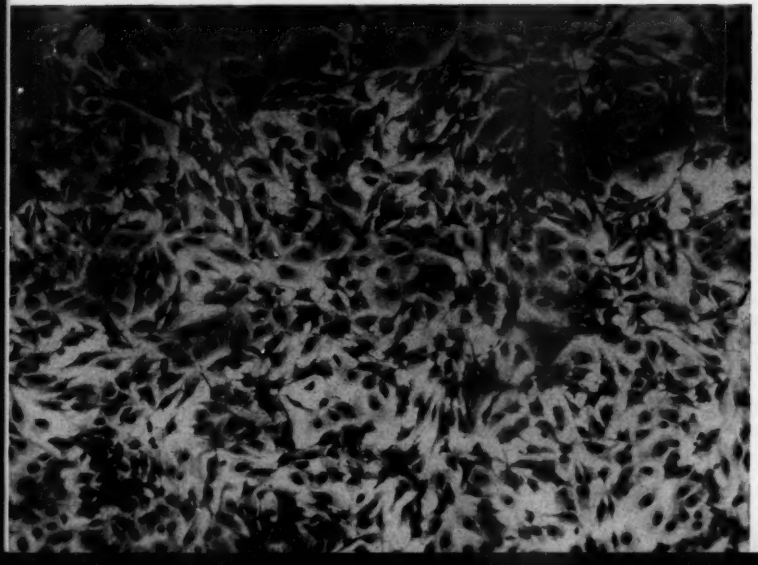

Fig. 2—Primary swine lung cell culture 4 days old. Notice the mixture of fibroblast and epithelial cells. Giemsa's stain; x 100.

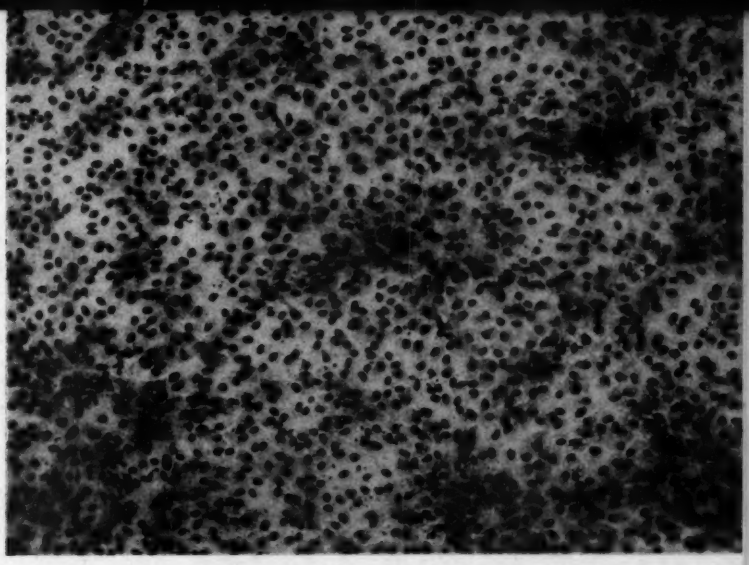

Fig. 3—Swine lung cell culture, 43rd serial passage. Giemsa's stain; x 100.

destruction by certain microorganisms was defined. The results are summarized (table 1), and the source of the microorganisms shown (table 2).

Information on the number of passages and detection of the microorganisms is given (table 3).

The swine nasal trichomonad and *Mycoplasma hyorhinis* were included in this study because these two agents are not eliminated from swine specimens by the routine antibiotic treatment and centrifugation procedures.

The nasal trichomonads reached maximal population density four or five days after introduction into the tissue cultures (fig. 10). By this time there was cell degeneration and a pH decrease of 0.2 to 0.4 units below that of the uninoculated control tubes. Cell degeneration was probably due either to exhaustion of nutrients or to accumulation of metabolic by-products and not due to direct action of the trichomonads on the cells. The trichomonads can be maintained by serial passage in the tissue cultures.

Mycoplasma hyorhinis is common in the nasal cavities of swine. In one initial trial, seven Selas No. 02 filter-passing cytopathogenic agents were isolated from 15 pools of swine nasal turbinates and tonsils. All seven of these isolates proved to be this organism. There was a slight lowering of pH due to the growth of this organism in the cell systems. However, the main action on the cells appeared to be damage of the

Fig. 4—Swine nasal mucosa cell culture, 39th serial passage. Giemsa's stain; x 100.

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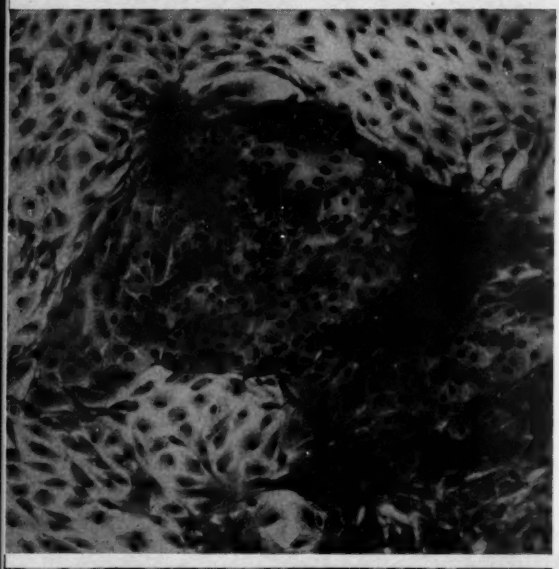

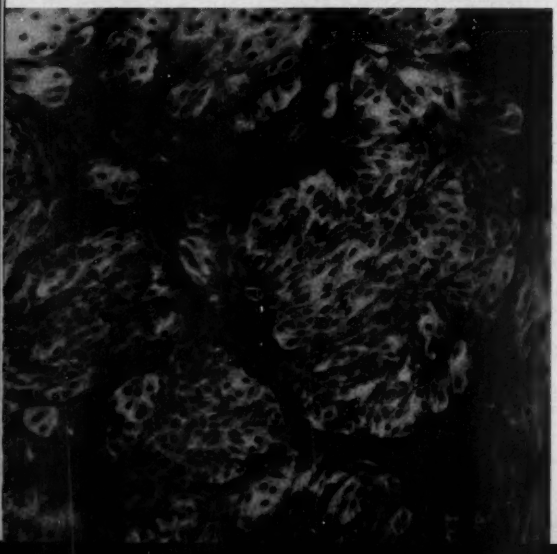

cytoplasm of the cell. The pleuropneumonia-like organisms were concentrated on the cell surface. After initial damage to the cell surface, they invaded the cytoplasm. The action on the cells could easily be mistaken for viral degeneration (fig. 8, 9). Giemsa-stained cover slip preparations of cells damaged by *M. hyorhinis* revealed numerous coccobacillary objects on the cell surface and in the damaged cytoplasm. Serial passage of isolates of this organism through 100 or more transfers in the ox heart infusion-chicken serum medium described by Switzer [17] did not destroy the ability of eight isolates of this organism to produce cell damage.

No damage detectable by direct bright-field observation or in Giemsa-stained cover slip preparations occurred following inoculation of three different hog cholera virus isolates into any of the cell systems used in this study, except in the primary swine lung cultures where a slight cytoplasmic vacuolization occurred. This change did not occur in two subsequent serial passages of the tissue culture fluid in swine lung cell cultures. A corresponding amount of normal swine serum produced the same slight cytoplasmic vacuolization. No attempt was made to detect hog cholera virus multiplication in the tissue culture systems.

All cell types inoculated with pseudorabies virus evidenced changes that were somewhat similar. The most extreme changes occurred in the serial passage and primary swine kidney cultures. In these two cultures, there was an initial rounding of cells detectable six to 12 hours after addition of virus. This change progressed for 24 to 36 hours until most of the cells were rounded and refractive. The cytoplasm of some of the cells increased four to six times their normal volume, and the nucleus enlarged. These cells had the appearance of distention due to imbibition of fluids. In addition, some of the rounded

Fig. 5(top)—Swine endothelium cell culture, 22nd serial passage. Giemsa's stain; x 100.

Fig. 6(center)—Swine endothelium cell culture with island of contaminating serial passage swine kidney cells. Giemsa's stain; x 100.

Fig. 7—Swine kidney cell culture, 42nd serial passage. Giemsa's stain; x 100.

TABLE 1—Effects of Certain Microorganisms on Swine Tissue Cultures

Microorganism	Serial passage cultures				Primary cultures	
	Kidney	Nasal mucosa	Lung	Endothelium	Kidney	Lung
Swine nasal trichomonad	+	+	+	N.D.	+	+
Mycoplasma hyorhinis	+	+	+	N.D.	+	+
Hog cholera virus	−	−	−	−	−	−
Pseudorabies virus	+	+	+	N.D.	+	+
Swine influenza virus	−	−	−	N.D.	+	+
Newcastle disease virus	−	−	−	N.D.	+	+
Eastern equine encephalomyelitis virus	N.D.	N.D.	N.D.	N.D.	+	+
Western equine encephalomyelitis virus	N.D.	N.D.	N.D.	N.D.	+	−
Virus pneumonia of pigs	−	−	−	N.D.	−	−

+ = cell destruction; − = no cell destruction; N.D. = not done.

cells clumped together. In stained preparations, multinucleated cell masses containing four to 12 nuclei were formed by coalescence of the cytoplasm of several cells.

Repeated attempts were made to obtain multiplication of swine influenza virus in serial passage cell cultures. Virus survival could be detected by inoculating embryonating chicken eggs, but no multiplication occurred. The swine serum used in these serial passage cell lines was found to be free of neutralizing antibodies against swine influenza virus. Attempts to grow swine influenza virus in primary swine kidney and swine lung cell cultures were successful. The chicken embryo-adapted virus produced cytopathic changes in the first series of swine kidney cells inoculated. The cell destruction continued through serial passage.

The primary swine lung cell cultures did not evidence definite cell destruction in the first virus passage, but developed mild evidence of cell damage by the third passage of the virus in this system. At this point, the swine lung cell passage virus was discarded and the swine lung cells inoculated with third passage swine kidney cell-propagated swine influenza virus. This produced severe cell destruction that could be serially passaged. Three passages through the more susceptible swine kidney cells had apparently allowed enough adaptation of the chicken embryo-passage swine influenza virus to the tissue culture system to make it pathogenic for primary swine lung cell cultures.

Another example of successful virus passage in primary cell cultures, but not in serial passage cell lines, was observed with Newcastle disease virus. Several different isolates of this virus failed to multiply in the serial passage cell line, even though the serum used in the systems had no detectable antibodies against this virus. On the other hand, the primary swine lung and kidney cell cultures allowed rapid multiplication of this virus with resultant cell destruction which continued through serial passage. The first evidence of cell damage was an involvement of the cell wall and cytoplasm giving some of the cells an irregular glistening appearance resembling a drop of melted fat. This stage was soon followed by fragmentation of the cytoplasm and nucleus. Complete cell destruction was usually present in 48 hours, although mature cell sheets required an additional 24 hours for complete destruction.

Eastern equine encephalomyelitis virus produced severe cell destruction in primary swine lung and swine kidney cell cultures. The cells became somewhat rounded in ap-

TABLE 2—Source of Microorganisms

Microorganism	Source
Swine nasal trichomonad	Nasal cavity of swine.
Mycoplasma hyorhinis	Nasal cavity and tonsils of swine.
Hog cholera virus	Isolates of three field strains supplied by Dr. L. H. Schwarte and Mr. John Mathews.
Pseudorabies virus	Rabbit brain passage Aujeszkey strain supplied by Dr. R. Shope. Swine kidney cell culture passage strain supplied by Dr. G. W. Beran.
Swine influenza virus	Shope Iowa 15 strain obtained as amnioallantoic fluid from Dr. M. S. Hofstad.
Newcastle disease virus	GB strain and two recent isolates supplied by Dr. M. S. Hofstad.
Eastern equine encephalitis virus	Commercial laboratory vaccine production strain.
Western equine encephalitis virus	Commercial laboratory vaccine production strain.
Virus pneumonia of pigs	Field strain passaged 2 to 7 times in respiratory disease-free pigs.

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pearance 24 hours after inoculation with the virus. The cell degeneration advanced rapidly until complete cell destruction had occurred 48 to 72 hours after inoculation. This effect was maintained through serial passage.

A somewhat different pattern of cell destruction occurred when western equine encephalomyelitis virus was inoculated into primary swine kidney and swine lung cell cultures. The swine kidney cells did not become as rounded as those infected with eastern equine encephalomyelitis virus and required about four days to undergo complete destruction. The swine lung cells were only moderately granular in appearance five days after inoculation. Serial passage of this swine lung tissue culture fluid failed to produce cell changes in swine lung cells.

Several attempts were made to produce detectable cell changes in the various tissue culture systems with virus pneumonia of pigs. In no case did serial passage of the tissue culture fluids produce cell changes detectable by direct bright-field examination or in Giemsa-stained cover slip preparations.

TABLE 3—Multiplication of Microorganisms in Swine Tissue Cultures

Microorganism	Number of serial passages in cell cultures: Serial passage: Kidney	Serial passage: Nasal mucosa	Serial passage: Lung	Serial passage: Endothelium	Primary: Kidney	Primary: Lung	Detection of microorganism
Swine nasal trichomonad	2	2	2	N.D.	5	4	Direct examination.
Mycoplasma hyorhinis	4	2	2	N.D.	3	3	Organisms recovered in ox-heart infusion-chicken serum medium from at least 1 passage in each tissue.
Hog cholera virus	3	3	3	3	3	3	Virus detection not attempted.
Pseudorabies virus	3	2	2	N.D.	6	3	10^{-6} t.c.i.d.$_{50}$ of virus present in sixth passage primary SK cell cultures. Similar undiluted material produced typical mortality in mice.
Swine influenza virus	3	3	3	N.D.	6	3	Serial passage SK, SNM, and SLu cell culture fluids neg. HA. No evidence of virus multiplication when these cell culture fluids were titered in chicken embryos. Primary SK and SLu cell culture fluids neg. HA, but had 10^{-4} to 10^{-5} titers upon titration in chicken embryos.
Newcastle disease virus	3	3	3	N.D.	40	3	Serial passage SK, SNM, and SLu cell culture fluids neg. HA. No evidence of virus multiplication when these cell culture fluids were titered in chicken embryos. Primary SK and SLu cell culture fluids neg. HA, but had 10^{-5} to 10^{-6} titers upon titration in chicken embryos.
Eastern equine encephalomyelitis virus	N.D.	N.D.	N.D.	N.D.	3	3	Virus detection in other systems not done.
Western equine encephalomyelitis virus	N.D.	N.D.	N.D.	N.D.	3	3	Virus detection in other systems not done.
Virus pneumonia of pigs	2	2	2	N.D.	2	2	Primary SK and SLu cell culture fluids inoculated intranasally and intratracheally into 4 to 6 pigs. The lungs of these pigs were normal four weeks postinoculation. Serial passage cell culture fluids not checked for the presence of virus.

N.D. = not done.

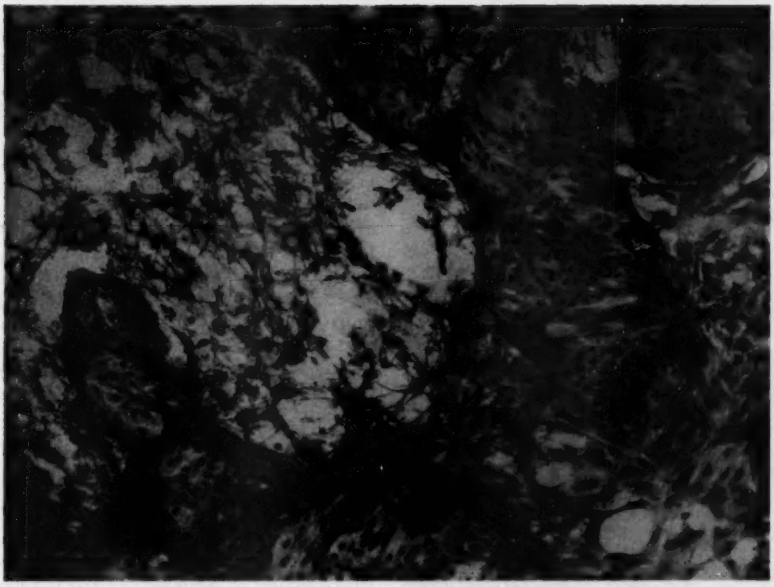

Fig. 8—Action of Mycoplasm hyorhinis on serial passage swine kidney cell culture 72 hours after inoculation. Giemsa's stain; x 100.

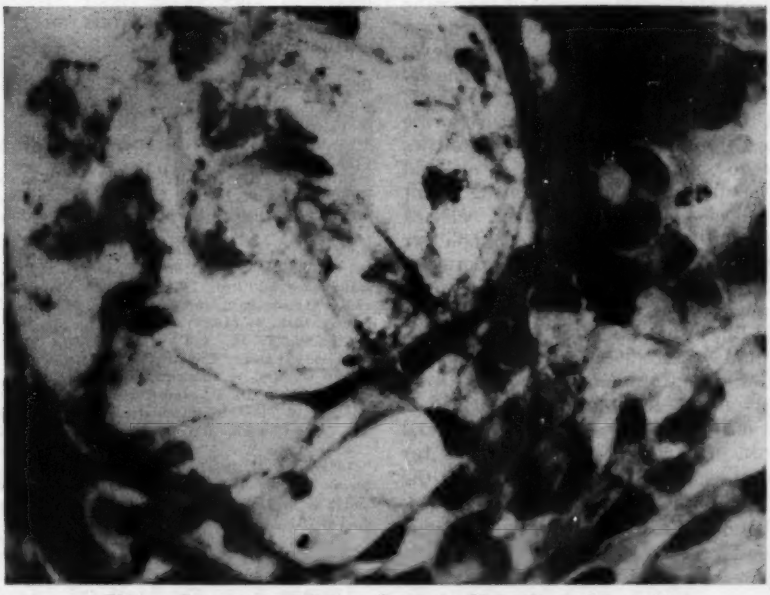

Fig. 9—Same preparation as figure 8. Giemsa's strain; x 430.

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Fig. 10—Four-day culture of swine nasal trichomonads in serial passage swine nasal mucosa cell line. Unstained; x 100.

DISCUSSION

The successful serial passage of the swine cell lines reported in this paper are believed to be due to several factors. A high-level swine serum medium was essential. The reduction of the serum level from 33 to 25 per cent reduced cell growth. Mature swine serum was found to have growth qualities superior to serum from 4- to 8-week-old pigs. The utilization of a chelating agent for cell sheet liberation produced cells of greater viability than did 0.25 or 0.33 per cent trypsin solutions. The incorporation of low-level trypsin (0.04%) in the solution for cell release was necessary because some cell lines were liberated by the chelating agent as small flakes of cells. It was found desirable to transfer the cell lines before a complete cell sheet was formed.

Three major problems were encountered in the use of serial passage cell lines for virus propagation. One of these was the lack of a suitable maintenance medium, although attempts were made to develop one. The second problem was the failure of certain viruses to grow in the serial passage cell lines even though they multiplied readily in the primary cell cultures. The third problem was the difficulty of maintenance of serial passage cell lines in a small laboratory operation during peak work loads. It is much easier to schedule work utilizing primary cell cultures. This is an important consideration for small laboratory operations.

The major advantage of the serial passage cell lines used in this work was that the cells were usually more uniform in appearance, with a relatively clear cytoplasm so that some stages of viral activity were more readily apparent. It is possible that certain serial passage cells will have increased susceptibility to some viruses, even though this was not the case in these studies.

SUMMARY

Serial passage cell cultures of swine kidney through 105, swine nasal mucosa through 87, swine lung through 65, and swine endothelium through 22 transfers are described. In addition, techniques for preparation of primary cultures from lungs and kidneys of 1-day to 8-week-old pigs are described. The effects of hog cholera virus, pseudorabies virus, swine influenza virus, Newcastle disease virus, eastern equine encephalomyelitis virus, western equine encephalomyelitis virus, virus pneumonia of pigs, swine nasal trichomonads, and *Mycoplasma hyorhinis* on these cell cultures are reported.

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Effect of Nitrofurantoin on Experimental Canine Bacterial Nephritis

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UNCOMPLICATED bacterial infection of the canine urinary tract is probably more common than is indicated by the frequency with which it is diagnosed. Early effective treatment is particularly important since, in many cases of canine nephritis, irreparable renal damage has been produced by the time the disease is discovered.[5] Pyelonephritis is found in about 5 per cent of all necropsies[2] and the importance of urinary infection as both a contributory[10] and complicating[3] factor in urolithiasis can hardly be overestimated.

During the past two years, clinical and laboratory aspects of canine urinary tract infection have been studied, and the effectiveness of various antibacterial agents including nitrofurantoin (Furadantin)* has been evaluated.[8] This report concerns experimental renal infections in dogs and the effect of oral treatment with nitrofurantoin.

MATERIALS AND METHODS

Twenty-nine dogs of common breeds, ranging from 6 months to 2 years old and weighing 15 to 50 lb., were used. The infection was produced by direct introduction of bacterial suspensions prepared from young cultures of pathogenic microorganisms into the renal pelvis or artery. Eleven strains of *Escherichia coli*, seven of Proteus sp., seven of *Staphylococcus aureus*, and one each of *Pseudomonas ceruginosa*, *Aerobacter aerogenes*, *Serratia marcescens*, and Streptococcus sp. (canine origin) were utilized.

Preliminary studies revealed that inoculation of bacterial cultures directly into the renal pelvis, medulla, or cortex usually failed to produce permanent infection. Only in dog No. 1 was infection established by this method. Of 28 dogs given the inoculum into the renal artery, nephritis was produced in 26. A surgical technique essentially identical with that used by early investigators[12] was utilized to produce infection.

The dogs were anesthetized with sodium pentobarbital and prepared for surgery in the usual manner. A 3-inch incision was made parallel to the last rib on the left side, through the skin, musculature, and peritoneum. The kidney was lifted into the incision and the fat dissected away from the proximal portion of the ureter to expose the renal artery. Forceps were placed under the vessel to make it easily accessible for injection. Varying quantities of bacterial suspensions, ranging between 0.5 and 1.0 ml., were injected through a 28-gauge needle. The amount of inoculum did not seem to affect the successful establishment of bacterial nephritis. All organisms used were of proven pathogenicity, except *S. marcescens*. Most of the strains had been isolated from dog or cats with clinical nephritis.

Urine samples from the normal dogs were collected and studied bacteriologically prior to inoculation, and urine specimens were collected within 24 hours after injection and at varying intervals thereafter. All specimens were collected by puncturing the bladder through the abdominal wall, utilizing a sterile syringe and 18-gauge, 1½-inch needle. As reported by other investigators,[4] this method is essential to prevent contamination by saprophytes from the urethra, prepuce, or vagina when collecting urine specimens from dogs.

Antibiotic sensitivity tests on all organisms were conducted as follows: One milliliter of a 4-hour broth culture of the organism was added to 4 ml. of melted tryptose-dextrose agar cooled to 45 C. This mixture was poured on the surface of a 5 per cent sheep blood agar plate, allowed to solidify, and discs* containing 100 μg. of nitrofurantoin were placed on the surface. After 18 hours' incubation at 37 C., the diameter of the zone of inhibition was measured. Sensitivity tests were carried out prior to inoculation and during and following therapy with nitrofurantoin.

Of the 27 dogs successfully infected in this series, 17 were treated with nitrofurantoin orally for six to 20 days. The daily dosage ranged from 150 to 600 mg. in four divided doses. A small amount of food was given with each dose. Treatment began five to 31 days postinoculation.

In addition to bacteriologic cultures of the urine, laboratory studies included urinalysis for protein, occult blood, glucose, and pH. Microscopic examinations of the sediment were made before and at various times during and after therapy. Blood samples were collected and complete hematologic studies were performed in conjuction with urinalysis.

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* Produced by Eaton Laboratories, Inc., Norwich, N.Y.

* Furadantin Sensi-discs, available from Baltimore Biological Laboratory, Baltimore, Md.

Results

The time lapse between inoculation and appearance of bacteria in the bladder urine was determined for all dogs. No apparent relationship between the bacterial species used and the rapidity of its passage into the urine was seen (table 1). In 18 of the experimental animals the organism appeared in the bladder urine 12 to 24 hours following inoculation. In a few cases, recovery of the organism was delayed. In dog 1, there was a 120-hour interval between inoculation of Proteus sp. into the renal pelvis and appearance of the organisms in the bladder urine. Another example of delayed recovery was in dog 10; *Esch. coli* was isolated from bladder urine 96 hours after inoculation. In dog 4, organisms of another strain of Proteus sp. appeared in the bladder urine two hours after being inoculated into the renal artery.

The results of nitrofurantoin treatment of the 17 dogs are shown (table 2). Failure to isolate the organisms from the inoculated kidney at necropsy occurred in only 3 of the 17 dogs. In 2 treated animals (dog 17, inoculated with *Ps. aeruginosa* and 13, inoculated with *Staph. aureus*) the organisms were isolated from both kidneys, indicating that the infection remained unilateral in the majority of the cases. The organisms were recovered during therapy on at least one occasion in all animals except dogs 14 and 1. In approximately 50 per cent of the animals, the organisms were isolated from all urine samples collected during treatment; in the others, sterile samples of bladder urine were obtained primarily after ten days of treatment.

The 12 untreated animals were necropsied at intervals ranging from four to 39 days following injection (table 3). The organism injected was recovered from the left kidneys and urine of 9 dogs. In 1 animal (dog 25) *Staph. aureus* was recovered from the urine and both kidneys. Two dogs were not permanently infected, as *Staph. aureus* was not recovered from either kidneys or the urine.

Since the organism remained in the urine throughout treatment, the total number of bacteria present at various times during treatment was determined (table 4). It is obvious from these data that the number of organisms decreased under nitrofurantoin therapy. The only exception occurred on the second day of treatment when the organism was present in numbers too numerous to count. The results of urinalysis remained relatively constant in this case, except the total number of leukocytes decreased with the beginning of treatment. The results indicate that nitrofurantoin was effective in eliminating the infectious agent in this animal.

The sequence of events in dog 10 is a typical progress report on the use of nitrofurantoin in the treatment of experimental nephritis (table 5). This dog was inoculated with *Esch. coli* isolated from a clinical case of canine nephritis. Original bacteriologic examination and urinalysis prior to inoculation failed to reveal any abnormalities. The organism was recovered from the urine four days following inoculation. Urinalysis revealed proteinuria and an increased number of leukocytes, but no casts. Similar findings prevailed until 14 days

TABLE 1—Hours Elapsing Between Inoculation of Bacteria into the Renal Artery and Their First Appearance in the Bladder Urine

Dog No.	Organism inoculated	Hours to urine
1*	Proteus sp.	120
2	Proteus sp.	72
3	Proteus sp.	16
4	Proteus sp.	2
5	*Esch. coli*	12
6	*Esch. coli*	15
7	*Esch. coli*	19
8	*Esch. coli*	48
9	*Esch. coli*	48
10	*Esch. coli*	96
11	*Esch. coli*	17
12	*Esch. coli*	24
13	*Staph. aureus*	48
14	*Staph. aureus*	16
15	*Staph. aureus*	16
16	*A. aerogenes*	20
17	*Ps. aeruginosa*	70
18	Proteus sp.	18
19	Proteus sp.	15
20	Proteus sp.	18
21	*Esch. coli*	48
22	*Esch. coli*	18
23	*Esch. coli*	18
24	*Staph. aureus*	20
25	*Staph. aureus*	18
26	*Staph. aureus*	....†
27	*Staph. aureus*	....†
28	*S. marcescens*	72
29	Streptococcus sp.	16

* In dog 1, the organisms were injected into the renal pelvis. † The organisms could not be recovered from the urine of dogs 26 and 27 at any time during the 14 days following inoculation.

XUM

after inoculation, when the number of leukocytes in the urine had diminished. Therapy was instituted four days later. Urinalysis throughout the period of treatment revealed few changes. Although the drug was administered following the feeding of a light meal, it was necessary to discontinue therapy after two weeks due to continual emesis. This animal was necropsied one week after cessation of treatment, and *Esch. coli* was recovered from the urine and the left kidney. The dog had received a total of 600 mg. of the drug daily for 18 days.

Typical results were obtained in dog 11, which was inoculated via the left renal artery with 1 ml. of a four-hour broth culture of *Esch. coli* obtained from a dog with clinical nephritis (table 6). The organism was isolated from the bladder urine on the day following inoculation and proteinuria was first demonstrated at this time. Casts appeared in the urine on that same day and were present throughout the course of the experiment. Therapy was instituted nine days after inoculation and consisted of 50-mg. doses of nitrofurantoin four times daily. No pathogenic bacteria were recovered from the urine on the third day of therapy. It was not possible to demonstrate the organism at any times during the period of treatment with nitrofurantoin. Large numbers of casts were constantly present in the urine. It is possible that nitrofurantoin existed in the urine at sufficiently high levels to inhibit the growth of bacteria. The drug apparently inhibited the organisms sufficiently to prevent their isolation during therapy, but on necropsy ten days after therapy was discontinued, *Esch. coli* was isolated from both the inoculated kidney and the urine.

Hemograms of both treated and control dogs revealed pronounced leukocytosis and a shift to the left immediately following surgery. This blood picture usually persisted throughout most of the treatment period. In some instances, however, institution of therapy was followed by a decrease in the total leukocyte count and a more normal distribution of the neutrophils. One feature was that the urea content of the blood did not increase, indicating that the normal kidney was capable of increasing its function to compensate for the inadequacy of its diseased mate.

Urinalysis revealed an increase in protein, erythrocytes, and leukocytes 24 hours following inoculation. The erythrocytes and protein generally disappeared within four days, whereas the leukocytes increased during this period. The number of leukocytes remained essentially the same throughout the experiment in untreated control animals. Some of the dogs treated with nitrofurantoin evidenced a drop in

TABLE 2—Time, Dosage, and Result of Nitrofurantoin Treatment of Experimental Canine Bacterial Nephritis in 17 Dogs

		Nitrofurantoin therapy			Recovery of organisms		
					During therapy		
Dog No.	Weight (lb.)	Initiated (days post-inoculation)	Duration (days)	Daily dose (mg.)	No. days recovered	No. days not recovered	Necropsy
1	40	21	6	300	0	1	Left kidney
2	15	5	4	150	2	0	
		9	4	300	1	0	
		13	6	300	0	1	Not recovered
3	25	23	20	200	10	0	Urine and left kidney
4	25	23	20	200	10	0	Urine and left kidney
5	25	22	10	200	5	0	
		51	10	400	3	3	Urine and left kidney
6	25	31	10	200	5	0	
		51	10	400	2	2	Left kidney
7	20	31	10	200	5	0	Urine
8	20	13	17	400	8	2	Not recovered
9	25	13	17	400	6	2	Not recovered
10	35	18	18	600	9	0	Urine and left kidney
11	25	16	19	200	2	5	Urine and left kidney
12	25	23	20	200	10	0	Urine and left kidney
13	18	21	19	200	2	3	Urine, both kidneys
14	20	10	20	200	0	8	Urine and left kidney
15	20	10	20	200	2	5	Left kidney
16	15	5	4	150	2	0	
		9	4	300	2	0	
		20	6	300	0	3	Not recovered
17	50	5	6	400	3	0	Urine, both kidneys

TABLE 3—Results of Bacteriologic Examination of Untreated Dogs

Dog No.	Weight (lb.)	Organism injected	Days elapsing before necropsy	Organism recovered at necropsy	
				Urine	Kidney
18	35	Proteus sp.	15	Proteus sp.	Proteus—left kidney
19	30	Proteus sp.	26	Proteus sp.	Proteus—left kidney
20	25	Proteus sp.	39	Proteus sp.	Proteus—left kidney
21	20	*Esch. coli*	12	*Esch. coli*	*Esch. coli*—left kidney
22	30	*Esch. coli*	22	*Esch. coli*	*Esch. coli*—left kidney
23	35	*Esch. coli*	39	*Esch. coli*	*Esch. coli*—left kidney
24	20	*Staph. aureus*	22	*Staph. aureus*	*Staph. aureus*—left kidney
25	20	*Staph. aureus*	24	*Staph. aureus*	*Staph. aureus*—both kidneys
26	20	*Staph. aureus*	20	None	None
27	25	*Staph. aureus*	20	None	None
28	20	*S. marcescens*	15	*S. marcescens*	*S. marcescens*—left kidney
29*	25	Streptococcus sp.	4	Streptococcus sp.	Streptococcus sp.—left kidney

* This animal died four days following injection and a purulent peritonitis was observed. Streptococcus sp. was isolated from the purulent material found in the peritoneal cavity.

TABLE 4—Urinalysis Data in Dog 8 as Observed 12 to 49 Days Following Inoculation

Days post-inoculation	*Esch. coli* cells/ml.	Albumin	pH	Occult blood	Sediment	r.b.c./field	w.b.c./field
12	25.2×10^5	1+	5.0	Neg.	Slight	0–1	Mod. clumps
13	(Nitrofurantoin therapy started)						
14	1.8×10^7	Neg.	7.5	Neg.	Slight	Rare	125–140
15	TC*	Neg.	8.0	Neg.	Slight	Rare	40–50
16	1,000	Neg.	7.0	Neg.	Slight	Rare	6–8
17	480	.....	....	.....	.....	.....	.....
19	1,000	Neg.	6.0	Neg.	Slight	Rare	5–7
20	88	.....	....	.....	.....	.....	.....
22	100	Neg.	7.0	Neg.	Slight	2–3	15–20
25	100	Neg.	7.0	Trace	Slight	0–1	2–3
29	17	Neg.	7.0	Neg.	Much	Rare	4–5
48	0	Neg.	6.0	Neg.	Much	0–1	3–5
49	0	Neg.	8.0	Neg.	Mod.	Rare	3–5

* Too numerous to count.

TABLE 5—Urinalysis Data in Dog 10 One to 43 Days After Inoculation

Days post-inoculation	Urinary organism	Albumin	pH	Occult blood	Sediment	r.b.c./field	w.b.c./field
1	None	Neg.	6.0	Neg.	Slight	None	0–1
2	None	Neg.	5.0	Neg.	Slight	None	4–6
5	*Esch. coli*	3+	5.0	+	Much	4–5	Many
6	*Esch. coli*	2+	7.0	+	Much	3–4	75–100
8	*Esch. coli*	1+	6.0	+	Much	Few	4–5
9	*Esch. coli*	Trace	8.0	Trace	Moderate	0–1	3–5
13	*Esch. coli*	Neg.	7.0	Trace	Much	1–2	40–50
14	*Esch. coli*	Neg.	6.0	Trace	Moderate	0–1	3–5
18	(Nitrofurantoin therapy started)						
19	*Esch. coli*	Neg.	6.0	Neg.	Much	Rare	6–8
21	*Esch. coli*	Neg.	5.0	Neg.	Slight	3–4	6–8
29	*Esch. coli*	Neg.	8.0	Neg.	Slight	Rare	2–3
35	*Esch. coli*	Neg.	7.5	Neg.	Slight	Rare	3–6
43	*Esch. coli*	Neg.	7.0	Neg.	Slight	Rare	3–4

TABLE 6—Urinalysis Data in Dog 11 from 12 Hours to 32 Days Postinoculation

Days post-inoculation	Urinary organism	Albumin	pH	Occult blood	Sediment	r.b.c./field	w.b.c./field	Casts
12 hours	None	....	7.0	Neg.	Slight	None	Few	None
1	*Esch. coli*	3+	8.0	+	Moderate	1–2	5–7	1+
3	*Esch. coli*	3+	7.0	+	Moderate		10–15	1+
4	*Esch. coli*	Trace	7.5	+	Moderate		3–4	1+
5	*Esch. coli*	Neg.	7.5	Neg.	Moderate	1–2	5–10	2+
9	(Nitrofurantoin therapy started)							
12	None	Neg.	8.0	Trace	Moderate	2–3	6–8	2+
18	None	Neg.	5.0	Trace	Moderate	3–4	6–10	3+
26	None	Neg.	7.0	Neg.	Moderate	1–2	8–12	1+
32	*Esch. coli*	Neg.	7.5	Neg.	Moderate	None	10–12	2+

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the number of leukocytes present within 24 hours after therapy was initiated.

Sensitivity tests of the organisms inoculated into the treated animals showed that all organisms except *Ps. aeruginosa* (dog 17) were sensitive to nitrofurantoin. The zone of inhibition around the nitrofurantoin disc varied from 10 mm. (dog 15) to 21 mm. (dog 7) in diameter. The average zone measured approximately 15 mm. before therapy. Sensitivity tests following therapy showed essentially indentical results in 16 instances. The only exception was in dog 3, inoculated with a strain of Proteus sp. This culture had a zone of inhibition of 11 mm. before therapy, and after therapy an apparently identical strain showed no zone of inhibition. This was the only instance in which the organism seemed to develop a resistance to nitrofurantoin. The zone of inhibition remained identical during and after therapy in all other animals.

DISCUSSION

The rapid passage of the bacteria through the kidney and into the urine in some of the cases is of particular interest. In animal 4, the organisms did not have time to produce sufficient renal damage to enable them to pass through the glomerulus. One would also question the possibility that bacteria could produce sufficient damage in an 18- to 24-hour period to allow passage into the urine. Had old cultures containing relatively large quantities of toxins been used, one would suspect that the toxins might cause sufficient injury to allow the organisms to pass through the glomerular barrier. However, since all the cultures utilized were four-hour broth cultures or saline suspensions of young cultures from an agar slant, it seems unlikely that toxins contributed to this phenomenon.

The results indicate that in a high percentage of the cases nitrofurantoin was unable to remove the organism completely from the kidneys following inoculation. In only 3 dogs (17.65%), known to be infected, was bacteriologic examination of the urine or kidneys negative at necropsy, even though treatment had been administered at relatively high dosages for periods up to 20 days.

It does seem, however, that nitrofurantoin can decrease the number of organisms to a point where they may not affect the over-all health of the animal. In some instances, pyuria, albuminuria, and other pathologic signs decreased during therapy. It was also noticed that in some treated dogs the hemograms returned to normal more rapidly than they did in the untreated control animals.

The degree to which these results can be applied in the clinic is unknown. Clinical experience [8] and available literature [1,9,7] indicate that in the usual case of canine urinary tract infection nitrofurantoin is likely to be beneficial.

One of the advantages of nitrofurantoin in the treatment of canine nephritis is the fact that most microorganisms do not develop resistance to the drug, even after long periods of exposure to it. Identical conclusions were reached in laboratory and clinical studies with nitrofurantoin in human medicine.[6,11,4]

SUMMARY

Experimental nephritis was produced in 27 of 29 dogs by inoculating pathogenic bacteria either into renal artery (26 dogs) or renal pelvis (1 dog). The organisms could be recovered from the bladder urine after two to 120 hours.

Nitrofurantoin was administered orally to 17 of these dogs, at doses ranging from 4 to 20 mg. per pound of body weight daily, for periods up to 20 days. The drug was first administered five to 31 days following inoculation. Sterile urine could be obtained in only 3 (17.6%) of these dogs. The drug was generally well tolerated, and there was little clinical or laboratory indication that most bacteria developed any significant degree of resistance to nitrofurantoin.

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An Epidemiological Study of Canine Lymphoma and Its Public Health Significance

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IT IS NOW 100 years since Leisering [5, 6] published the first report of the occurrence of leukemia in lower animals (horse). His report followed 13 years after the discovery in 1845 [4, 5] of leukemia as a separate disease entity in man. A century of study has revealed the widespread distribution of this disease among the mammalian and avian species. Feldman [6] stated, "If all species are considered, the lymphoblastomas probably occur with greater frequency than any other neoplasm which affects lower animals."

The distinct histopathological resemblance of this disease in lower mammals to that in man has spurred the study of the pathogenesis of leukemia-lymphoma in all animals over the years. Of recent interest is the application of the epidemiological method to the study of the character and distribution of this disease. Based on census, death certificates, and cancer registry data, the following pattern of lymphoma-leukemia in man has emerged:

In the United States, neoplasms of the lymphatic and hematopoietic tissues account for approximately 1 per cent of deaths from all causes and 8 per cent of deaths from neoplastic diseases.[11] In 1953, the over-all crude death rate was 12.5 per 100,000 population. The rates are higher among males than females. The age distribution of death rates from lymphatic and acute leukemia is bimodal, having an early peak in the first two decades of life and rising again in the sixth and seventh decades, as opposed to myelocytic leukemia which is more common than the lymphatic form in the third, fourth, and fifth decades. A bimodal age distribution was noticed by MacMahon [8] in his study of the epidemiology of Hodgkin's disease. He reported one peak of incidence in the third decade and a second at the eighth. Meadors [9] noticed a decided urban distribution of leukemia with consistently higher age-specific rates for urban areas than could be accounted for by superior diagnostic services in the cities. Sacks and Seeman [10] showed that death rates from leukemia increased with corresponding increase in average rentals.

COMPARABLE DATA IN ANIMALS

What comparable data do we have in animals? Although we have known of this disease in our domestic animal populations for 100 years, we have been only able to guess at its true incidence and distribution. The adaptation of the epidemiological method to this problem lacks a base line of population data which is to be found in the census tracts of human populations. Death certificates as a source of information on primary cause of death are not available in veterinary medicine and the case records maintained by most veterinary institutions and private hospitals lack the uniformity of a standard nomenclature and are not reportable to a central statistical agency such as the National Office of Vital Statistics.

In general, veterinary data are obtained from condemnations from meat inspection activities, special tumor collections, and reports of cases from individual veterinary practitioners or hospitals. These data often

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lack histopathological confirmation, adequate descriptions of the age, breed, and sex of the animals involved; the geographic area and population from which they originate; or the specific time intervals involved.

Of particular interest is the reported occurrence of malignant lymphoma in dogs. In 1931, Wirth,[3, 5] in Vienna, found 10 cases in 2,763 dogs brought to a clinic, an occurrence rate of 0.36 per cent, among which males predominated. Only adults (over 2½ years old) were affected. In 1945, Bloom and Meyer [1] in New York City reported 20 cases in 10,000 dogs, an occurrence rate of 0.2 per cent. Twelve of the animals were males, and 7 of the 20 were Scottish Terriers. Ages ranged from 5 to 12 years. In a review of three collections of neoplasms which included 5,315 specimens from dogs in England, South Africa, and the United States, Smith and Jones [12] reported 181 specimens of malignant lymphoma, or 3.4 per cent of the total collection. For the reasons mentioned, reservations must be expressed as to the relationship of this type of data to true incidence. As Gilliam [7] pointed out, incidence includes an implication of risk for which we must have (1) the number and description of the population at risk, (2) the number and description of cases involved, and (3) a specific time interval.

THE NEW JERSEY STUDY

It was with these limitations of presently available veterinary data in mind that a Public Heath Service veterinarian was assigned to the Cancer Control Program from the Bureau of Veterinary Public Health of the New Jersey State Department of Health. The problem was to develop, as part of a joint study, epidemiological methods for obtaining information on the incidence of malignant lymphoma in lower animals which would be of value to current epidemiological studies of lymphoma-leukemia in man.

A factor of interest in the study of lymphoma in man is the contribution which environment makes to the cause of this disease. The evaluation of the role of environmental factors in human lymphoma presents certain problems. How do we define the environment of man? Most human beings spend 11 hours a day going to, from, and at work. They frequently move several times in their lifetime and even the children attending school are gone six to seven hours daily. These problems are minimized for pets. They are usually confined to their specific environment and their shorter life span makes the definition of their specific environment at the onset of a chronic disease a more simple task. The similarity of the disease process in man and lower animals makes it reasonable to assume a similarity of inciting agents. Based on this assumption, the pet may serve as a more reliable indicator of environmental lymphogenic factors. A study was designed which would establish a lymphoma registry for man and dogs on a state-wide basis. The environmental distribution of cases would be studied for areas of consistently high incidence for dog and man.

BASE LINE POPULATION DATA

In the establishment of a canine lymphoma registry, two major problems were encountered: (1) the securing of base line population data, and (2) the establishment of a reliable case report system.

To obtain base line population data, a survey was made of state canine registration records. In 1957, New Jersey registered 385,427 dogs in accordance with its Rabies Control Program. Annual registration is required of all dogs ½ year of age and older. Registration requirements necessitate the obtaining of age, sex, and breed information on each of the dogs registered. These data must be kept for three years by the local municipalities according to state law.

For purposes of this study, the state was divided into eight socio-economic areas according to the method of Brush.[2] Areas 1 and 2 were densely populated metropolitan areas adjacent to New York and Philadelphia, respectively. Areas 3, 4, and 5 were northern, central, and southern agricultural areas. Areas 6A and 6B were northern and southern seashore resorts, and area 7 was a sparsely settled, wooded, rural area known as the Pine Belt.

The number of dogs registered in each area is shown (table 1). The number of persons per registered dog for the state as a whole was 14.3, or approximately 1 dog for each four households (using 3.5 as the average number of people per household for New Jersey as obtained from U. S. census data). In the rural areas of the

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state (areas 3, 4, 5, and 7) there was approximately 1 dog per two households, a greater number of registered dogs per unit of human population.

The survey procedure employed was to select 40 communities throughout the state. Municipalities selected were chosen with a view toward geographic distribution within the socio-economic areas as well as to the adequacy of registration records within the communities. A complete census was performed of the 1957 canine registration records of these 40 communities. The number of communities selected within each area was further governed by the desire to include approximately 15% of the total canine registration in the survey. From this point on, the communities selected will be considered as representative of the socio-economic area from which they came and their total will be considered as representative of the state total.

The number and percentage of canine records surveyed in each area are shown (table 1). A total of 56,663 records (14.7% of the dogs registered in the state) were surveyed from areas containing 20.4 per cent of the human population which in 1956 yielded 19.6 per cent of the human deaths due to lymphoma-leukemia. Of these, 1,061 were found to be incomplete and had to be discarded, leaving a total of 55,602 complete records for analysis.

Age data were adjusted in order to more accurately determine the median age. Since registration begins at ½ year of age, it was assumed that doubling the 0 year data would conservatively (due to high pup mortality) indicate the number of dogs which might have been registered had registration been required at birth.

The median age for all dogs was 3.7 years, and 9.7 per cent of the dogs were 10 years of age and older (table 2). With

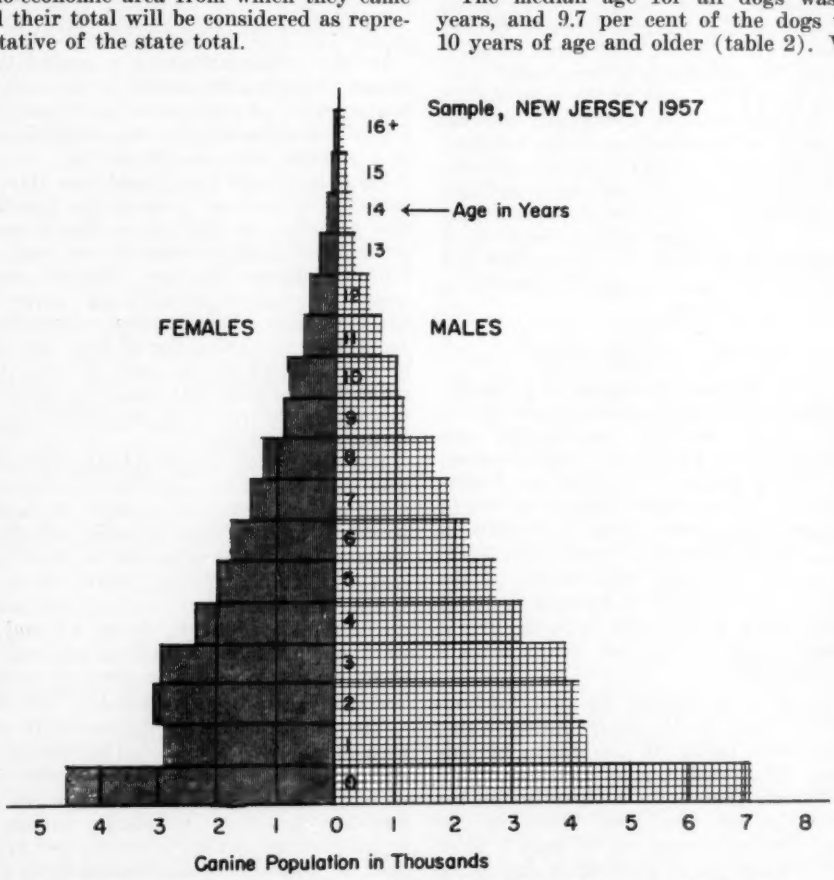

Fig. 1—Distribution of the canine population of New Jersey in 1957 by age and sex.

the exception of the 0- and 1-year age groups, there was a notable uniformity of percentages per age group for the various areas. Some variations from the state average were noticed, particularly in areas 4 (central agricultural), 6B (southern seashore), and 7 (Pine Belt) which will be followed in future surveys for significance.

There were more males than females in the population surveyed (fig. 1). This was probably due to the preference of pet owners for male dogs and will be investigated further. That it was not due to a trend in mortality was indicated by the fact that the median age as well as the percentage of animals over 10 years old was slightly, but not significantly, larger in females than males. In females, the median age was 3.8 years and the percentage of animals 10 years of age and over was 9.9 per cent. In males, the median age was 3.6 years and the percentage 10 years of age and over was 9.5 per cent.

TABLE 1—New Jersey Canine Registration Survey, 1957

Area	Dogs registered per area	No. of communities surveyed per area	Records surveyed per area	Total records in area (%)
1	212,266	9	30,820	14.5
2	59,173	6	8,980	15.2
3	32,034	6	4,618	14.4
4	17,095	4	2,345	13.7
5	25,985	5	3,901	15.0
6A	22,396	3	3,573	16.0
6B	7,230	3	1,112	15.4
7	9,248	4	1,314	14.2
Totals	385,427	40	56,663	14.7

A total of 110 breed descriptions was tabulated from the survey. The 20 listed (table 3) accounted for 90 per cent of the records surveyed. The breed distribution was also acquired for each of the eight areas. Mixed breeds made up approximately one-third of the registered dogs in the state. Cocker Spaniels were the most popular specified breed, accounting for approximately 10 per cent of the population. They tended to be more popular in the urban areas of the state. Collies and hounds were frequently found in the rural areas. In a dog population suffering from a ubiquitous disease, we would expect a greater number of clinical cases to occur among the more popular breeds.

It has been said that larger breeds of dogs do not live as long as smaller breeds and that a Great Dane over 10 years of age is a veteran. Our data support this view. Larger breeds had a lower percentage of survival over 10 years of age (Great Dane, 5%; Collie, 5.4%; and German Shepherd Dog, 7.9%) than did smaller breeds (Cocker Spaniel, 9.8% and Fox Terrier, 17.8%).

TABLE 2—Age of Dogs Per Area

Area	No.	Median age (years)	10 years & over (%)
1	38,830	3.6	9.5
2	9,668	3.8	10.7
3	4,968	3.6	8.7
4	2,410	4.2	9.5
5	3,888	3.7	9.0
6A	3,965	3.4	9.0
6B	1,236	4.3	13.2
7	1,411	3.7	7.8
Totals	61,376	3.7	9.7

This type of data will, we hope, establish the basis for making generalizations as to the age, sex, and breed distribution of dogs in various types of communities within the state. Similar census surveys at three years (1960) and five years (1962) hence will be performed. They will establish population trends and maintain the base line over the five-year period. Several additional factors must be evaluated; *i.e.*, what is the extent of veterinary coverage in these communities, how many and what kind of animals do they see, what is the turnover of dogs per community, how many move in or out, how many stray, how many are killed, how many die, etc. In this regard, it would be of tremendous value to follow a particular age cohort through a five- or ten-year period. It would also be important to know the ratio of registered to unregistered dogs.

CASE REPORT SYSTEM

Against this population background must be established a case report system which will supply to a central registry the confirmed cases of lymphoma. Two self-addressed, stamped, specimen kits containing specimen jars with formalin for biopsy or necropsy material, glass slides for a peripheral blood smear, and a questionnaire for animal identification were distributed to cooperating veterinarians throughout the state. To date, 130 veterinary hospitals are participating in the project.

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The procedure begins with the client who brings a suspect animal to the veterinarian. A biopsy or necropsy specimen is taken and mailed with the completed questionnaire to the Bureau of Pathology of the State Department of Health, where slides are prepared by the histologist (Dr. E. O. Gilbert) and forwarded to a veterinary pathologist (Dr. J. R. McCoy) at the Bureau of Biological Research, Rutgers University. The pathologist sends his report directly to the practicing veterinarian who then reports to his client. This free service has been established to yield a definitive diagnosis to the practitioner within a week or ten days from the time of submission of samples. A copy of the report plus the questionnaire is returned to the veterinary epidemiologist in the Bureau of Veterinary Public Health of the State Department of Health. The epidemiologist then contacts both the practitioner and the client for a follow-up. He is also responsible for liaison with the Bureau of Cancer Control of the State Department of Health engaged in the human lymphoma-leukemia studies. Statistical and pathological consultants inside and outside the Department are utilized by personnel involved.

TABLE 3—Breed Distribution of Dogs From 55,602 Canine Registration Records Surveyed, New Jersey 1957

Breed	Total in Survey	
	No.	%
Mixed breed	20,621	37.1
Cocker Spaniel	5,595	10.1
Collie	3,066	5.5
Fox Terrier	2,901	5.2
Beagle	2,799	5.0
Boxer	2,679	4.8
German Shepherd Dog	2,435	4.4
Terrier (Unspecified)	1,918	3.4
Hound	1,852	3.3
Dachshund	1,008	1.8
Spitz	975	1.8
Boston Terrier	745	1.3
Toy Fox Terrier	663	1.2
Dalmatian	484	0.9
Chihuahua	409	0.7
Irish Setter	408	0.7
Doberman Pinscher	400	0.7
Poodle	384	0.7
Wire Haired Terrier	366	0.7
Springer Spaniel	359	0.7
Other	5,535	10.0

DISCUSSION

This project will develop in the next few years to a point where it should annually yield approximately 200 cases and give adequate numbers upon which to base conclusions. With the added information from the three- and five-year census survey data we should like to be able to develop age, breed, and sex-specific death rates and use a modified life table approach to the determination of incidence rates for specific communities. This may locate specific areas of high incidence for further study.

In performing this study of the magnitude and distribution of lymphoma in dogs we shall, in addition, be obtaining clinical and laboratory specimens en masse for studies of the cause, pathogenesis, and therapeutic response. Statistical evidence will be sought regarding transmission from animal to man and vice versa.

The large number of New Jersey veterinary hospitals participating enables us to present the group experience and to avoid the pitfalls which the "clinical impression" of a single practice might present. The veterinary profession thus participates directly in cancer research and provides information which will aid in the solution of a major public health problem.

In his book entitled "Leukemia in in Animals," Englebreth-Holm [5] concludes "With the solution of the problem of human leukemia as the final goal, the study of the nature of the disease and of its various features in animals must therefore be regarded as abundantly justified, since the results attained are likely at the same time to elucidate the corresponding features of leukemia in man."

SUMMARY

This report describes a field study currently being undertaken throughout the State of New Jersey. The study represents the first establishment of a canine lymphoma registry on a state-wide level.

Base line population data of dogs as to age, sex, and breed are presented for the various socio-economic areas of the state. These data were obtained from an analysis of a sample of 55,602 records selected from 385,427 dogs licensed in 1957 in New Jersey.

A case report system is described which will yield epidemiological information as to the magnitude and distribution of this disease in dogs within various communities of the state and perhaps serve as an indicator of the presence of environmental and other factors of significance in the etiology of the disease in animal and man. It will also serve as a source of raw data for

laboratory studies in the etiology, pathogenesis, and therapy of malignant lymphoma.

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The Oral and Pharyngeal Pathology of Domestic Animals
A Study of 487 Cases

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THIS IS A SECOND study, undertaken to ascertain types and frequency of oral and pharyngeal lesions occurring in a large number of domestic animals. Gorlin *et al.*[43] presented a smaller series in 1958.

Occasional case reports of oral lesions in domestic animals are available, but other general surveys of both neoplastic and non-neoplastic oral lesions are lacking. Only limited conclusions should be drawn from compilations of case reports, as there is a tendency to report the unusual while ignoring common or self-limiting lesions, *i.e.*, viral papillomatosis. Moreover, a survey in one part of the country may differ from that in another part, with even greater variation in different parts of the world. Difference in species distribution may account for this variation, but other factors probably are involved also.

There seems to be little justification for comparing the statistical data obtained from animals with those from man. The samples of human oral pathology cited by Woodbridge,[128] Boyle,[16] McCarthy,[66] and Gorlin,[39] as compared with the current study, show wide disparity between the frequency of oral lesions in man and other animals. Comparison of natural histories of similar morphological and etiological entities among the various species and man appears warranted.

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Clinical or pathological follow-up is usually far more difficult to obtain for animals than for man. The specimens from animals suffer from all of the inadequacies of those from man, frequently being poorly documented, improperly fixed, or inadequate in size and representation, or worse.

MATERIALS AND METHODS

The materials for this study are from the files of the Registry of Veterinary Pathology of the Armed Forces Institute of Pathology, Washington, D.C., and the Surgical Pathology Division of the School of Veterinary Medicine, University of Minnesota. All cases with lesions of the oral cavity, oral pharynx, and perioral areas were screened and only those with biopsied lesions from these areas were retained. The resultant series contained 487 cases, consisting of 377 dogs, 44 cattle, 35 cats, 17 horses, 9 mules, 3 sheep, 1 burro, and 1 pig. Routinely, the materials had been fixed in formalin, embedded in paraffin, and stained with hematoxylin and eosin. Various special staining techniques were utilized when considered necessary or contributory.

OBSERVATIONS

For the sake of clarity, the material is presented under the broad headings of *(a)* non-neoplastic lesions (table 1); *(b)* benign neoplastic lesions (table 2), and *(c)* malignant neoplastic lesions (table 3). Although our categories are not rigid, we have attempted to stay within this framework. Discussion and review of the literature are generally incorporated under the pertinent headings and subheadings and are limited largely to those pathological lesions represented in our series.

NON-NEOPLASTIC LESIONS

All included lesions not considered neoplastic constitute approximately 28 per cent (135) of our 487 cases. Numerous systemic diseases of animals with oral manifestations are not represented in our series, for many of these such as blacktongue (canine), bluetongue (ovine), aphthous

fever or foot-and-mouth disease (several species), bovine malignant catarrh, rinderpest, mucosal disease complex (bovine), etc., are not ordinarily biopsied and hence would not come under our purview.

An excellent discussion of the differential diagnosis of these and other similar diseases can be found in Smith and Jones.[111] However, considerable work is needed to establish the nature and pathogenesis of the various lesions and what histological features might be of diagnostic importance.

effect of ammonium carbonate produced from salivary urea. Bliss[11] succeeded in reproducing the condition in normal dogs by applying saliva from nephrectomized dogs to normal oral mucosa. Canine leptospirosis and pellagra are also associated with nonspecific oral ulceration. It is likely that kidney dysfunction plays a role in the oral lesions of these diseases also.

Foreign Body and Granuloma.—Both intrinsic foreign bodies such as calcalus of salivary gland or duct, and extrinsic ob-

TABLE 1—Non-Neoplastic Lesions of Domestic Animals

Lesions	Canine	Feline	Equine	Bovine	Other
Nonspecific inflammation	39	10	1 horse 1 mule	2	1 porcine
Pyogenic granuloma	11	10	..	1	..
Foreign body and/or FBG	5	2	2 horses 2 mules	1	..
Giant cell reparative granuloma-like lesion	1	..	..	..	..
Actinomycosis	..	..	..	13	..
Actinobacillosis	..	..	..	7	..
Trichinosis	1	..	..	..	..
Globidiosis	..	..	1 burro	..	..
Hyperplasia	1	..	..	..	..
Eosinophilic granuloma	..	2	..	..	..
Cyst					
Salivary gland	9	..	..	..	..
Gingival	1	..	..	..	..
Dentigerous	1	..	..	..	..
Branchiogenic	2	..	..	..	..
Teratoma and Odontoma	3	..	..	1	1 ovine
No diagnosis (histiocytomas?)	2	..	..	1	..
Total	76	24	3 horses 3 mules 1 burro	26	1 porcine 1 ovine

Nonspecific Inflammation.—Of the 135 non-neoplastic lesions, 54 (40%) exhibited only banal inflammation, especially of the lips, tongue, gums, or tonsils. Special note was not made concerning the degree of chronicity or severity of the lesions nor were any special organisms demonstrated. Almost three fourths of these lesions were from dogs and well over half (most of them ulcerative) were associated with the terminal phase of kidney dysfunction. It is possible that this clinical association is so commonly recognized that, like canine oral papillomatosis, oral lesions are seldom biopsied. The significance of these lesions in the other cases was not established, but in domestic animals there are many diseases with oral lesions for which a diagnostic microscopic picture has not been established.

The association of membranous or ulcerative stomatitis with uremia is occasionally observed in man also. Black[9] considered this stomatitis to be due to the caustic

jects, such as splinters and oat awns, are included within this category. Five were found in dogs; 2 each in cats, mules, and horses; and 1 in a cow. Plant husks and fibers seemed to be the chief offenders. Other objects such as bones, sticks, and stones which become wedged between the teeth are not included.[101]

Pyogenic Granuloma.—There are 22 cases of pyogenic granuloma in our series. The gingiva was a common site. Eleven occurred in dogs, 10 in cats, and the remaining 1 in a 3-day-old calf. Neither age nor breed seems to be a factor. Although our series is too small to reflect the true incidence, it represents approximately 29 per cent of all oral lesions and 42 per cent of all inflammatory lesions in the cat. This lesion is said to be especially common on the oral mucosa of cats and has been erroneously called "rodent ulcer."[67] It has been shown to be nonviral in origin. Several bacteria (*Bacillus proteus, Staphylococcus aureus,* and *Streptococcus hemo-*

XUM

lyticus) have been implicated as etiological factors.[96] It represents proliferation of granulation tissue in response to low-grade chronic infection or hormonal stimuli, or both. In an overwhelming majority of cases, the disease is spontaneous although occasionally two or more animals in the same household are affected and sometimes "kissing" lesions are observed involving two approximating surfaces.

The clinical appearance varies with the age of the lesions and the degree of ulceration or epithelization or both. As in man, the pyogenic granuloma is generally brownish red with a granular surface. In older, well-epithelized lesions, the color is less red and the surface is rather smooth.

Histologically, the pyogenic granuloma in man and domestic animals is the same. The lesion is composed of dilated blood capillaries, fibroblasts, and a variable number of inflammatory cells (fig. 1).

Actinomycosis and Actinobacillosis.—Thirteen cases of actinomycosis, all in cattle, are represented in our series. The disease is reported occasionally in the mouths of animals other than cattle.[69, 71, 103] Swine, although manifesting the disease systemically, seldom have oral involvement.

Actinomycosis is caused by the ray fungus, *Actinomyces bovis*. The organism is thought to enter the jaws by way of abrasions of the oral or phyaryngeal mucosa due to the rough nature of the diet. It produces an enlargement of the affected maxilla or mandible called "lumpy jaw." The infection may spread to the deeper structures of the neck or even the cranial fossa but does not usually involve lymph nodes. In contrast with man, viscera are seldom involved, although in 1 case in our series the tongue was affected. In the older literature, involvement of the tongue, with subsequent induration called "wooden tongue" was attributed to this fungus. However, investigation by Lignieres and Spitz[65] showed that this condition was caused by *Actinobacillus lignieresi*.

In actinobacillosis, lymph nodes, especially about the head and neck, are commonly involved, as well as skin, mucosa, and internal organs. In contrast to actinomycosis, bone is apparently never attacked. Occasionally, both organisms are found in the same animal. Seven cases of actinobacillosis in cattle are included in our series, 3 with involvement of the tonsillar and pharyngeal areas.

Both diseases are characterized by abscesses containing small grayish white or yellowish granules. Microscopically, these consist of rosettes of radiating clublike structures. In actinomycosis, the central area of the rosette consists of gram-positive filamentous mycelia, while in actinobacillosis it is composed of minute gram-negative nonbranching coccobacilli. The club-shaped terminal ends of both colonies are quite similar. The "clubs" are probably produced by the host and surround the colony but are not related to any individual organism. The organisms of these two diseases differ materially in their cultural characteristics. In contrast to most other granulomatous diseases, the cellular response immediately about the organisms consists chiefly of polymorphonuclear leukocytes.[108] Because both organisms are easily stained with aniline dyes, especially carbolfuchsin, Gram's stain should always be employed for differentiation. An especially good stain that can be used with

Legend for Plate 1 on Opposite Page

Fig. 1—Canine pyogenic granuloma. Note large number of endothelial-lined vascular spaces. In the edematous stroma are a number of histiocytes.

Fig. 2—Feline eosinophilic granuloma. Note large numbers of eosinophilic leukocytes exhibiting markedly granular cytoplasm. Intermixed are numerous histiocytes.

Fig. 3—Bovine sarcosporidiosis. Longitudinal section of muscle exhibiting elongated tubule, characteristic of Sarcocystis colony. Spores may be seen as banana-shaped structures within cysts.

Fig. 4—Equine globidiosis. In contrast to Sarcocystis, the organism, Globidia besnoiti, a hyalinized capsule around the colony, is exhibited. The spores are smaller than those seen in figure 3.

Fig. 5—Canine trichionosis. Cross-section of tongue muscle, showing encysted larval form of Trichinella spiralis. Note hyaline capsule.

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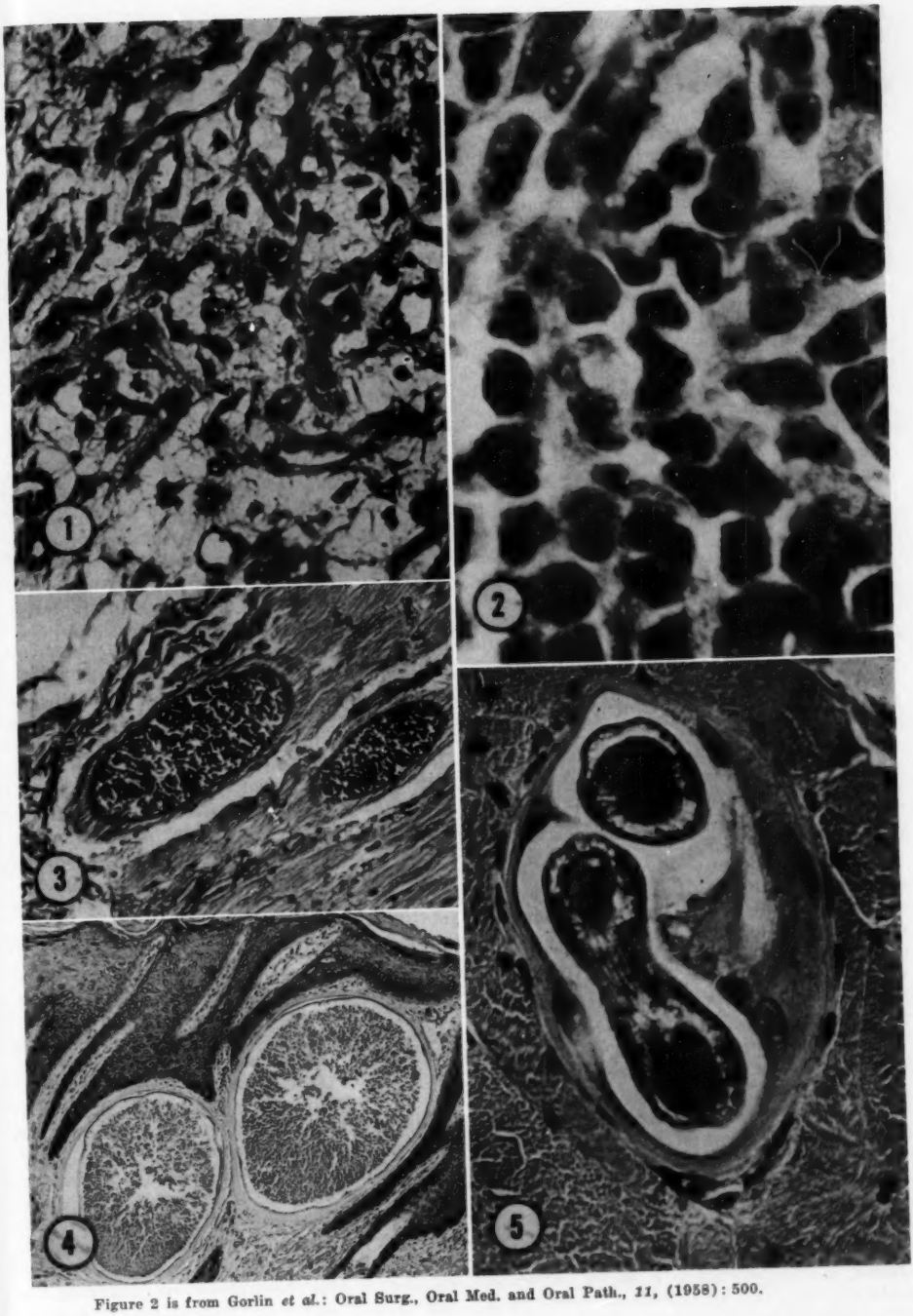

Figure 2 is from Gorlin *et al.*: Oral Surg., Oral Med. and Oral Path., *11*, (1958): 500.

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facility is that devised by Brown and Brenn.[17]

Giant Cell Reparative Granuloma.—The giant cell reparative granuloma seldom occurs in animals. The lesion comprises about 1 per cent of human oral pathological changes. Only a single case occurred in our series. The lesion was located in the gingiva of a 10-year-old Dalmatian dog. Olafson [81] described another in a 6-year-old Bulldog. Microscopically, the animal and human lesions are somewhat similar, multinucleated giant cells being seen in a very cellular vascular stroma.[122]

Eosinophilic Granuloma.—Two such lesions were found, 1 in the upper lip of a 3-year-old female cat and another in an 11-year-old male cat. No bone lesion was observed. Whether this lesion is specific for cats is not known. The microscopic picture was similar to that seen in man. Lever [62, 64] has pointed out that in man several distinct types of eosinophilic granuloma occur. There is the "true eosinophilic granuloma of the skin" which is simply a dermal manifestation of the mildest form of osseous cholesterol histiocytosis. A second type is not associated with bone involvement and is designated "eosinophilic granuloma of the skin, erythema elevatum diutinum type." Lesions are frequently manifest on the face.

The microscopic picture is characteristic. There is a dense granulomatous infiltration, especially around capillaries, consisting largely of eosinophilic leukocytes and histiocytes (fig. 2).

Parasites.—Because of its apparent monotonous frequency in the skeletal muscle of so many species and lack of pathological response, we have not recorded the incidence of sarcosporidiosis. If serial sections were made of the tongue, especially of herbivores, it would, no doubt, be one of the commonest lesions [107] (fig. 3).

A burro with globidiosis of the lip represents the single case of protozoal infection specifically recorded in our series. The etiological agent, variously referred to as *Globidium besnoiti* and *Besnoitia besnoiti*, is responsible for a chronic cutaneous and systemic disease of cattle and horses.[54, 95] Jones [111] states that it is also found in Mexican burros which are not apparently systemically affected. Microscopically, this disease is characterized by large spherical cysts with a thick hyaline wall lined by a thick monolayer of large nuclei and cytoplasm and filled by small elongated spores about half the size of those of sarcocystis (fig. 4).

The other parasitic lesion in our series was that of trichinosis in a dog. The larval form of *Trichinella spiralis* was encysted in a skeletal muscle fiber of the tongue and did not differ significantly from the appearance in other animals (fig. 5).

Neoplasms.—Of numerous case reports of neoplasia in domestic animals,[25, 27, 28, 34, 73, 94, 100, 119, 126] relatively few relate to oral tumors.[15, 43, 49, 64, 104]

Many factors enter into apparent species incidence in addition to actual species difference. One must consider an "endearment factor"—the intimacy of daily contact where closer observation of the deviation from the norm is possible. The relative population of the various species may be reflected in any series. While these may be partially explained by the overwhelming number of specimens in our series from dogs, other factors must be considered, since cats are probably more numerous than dogs.[1] An important factor is the slaughtering of many domestic animals before they reach "cancer age." [91] Trotter [120] did not report any neoplasms in 5,300 slaughtered sheep 6 to 12 months of age. The source of materials plays a major role in any collection of tumors. In three papers, Plummer [93, 94] presented a series of over 700 tumors from domestic animals. In his most recent publication (1956), of 636 tumors, 447 were bovine and 83 were canine. That the vast majority of specimens were submitted to the Canadian Department of Agriculture by veterinarians stationed at abattoirs may explain this apparent incongruity. Biased collection of materials may affect not only species proportions but relative proportions of lesions in a given species.

In our series, the most common benign and malignant canine oral neoplasms are epulis and melanoma, respectively. Perhaps the true incidence of infectious canine oral papillomatosis (24 of 146 benign oral tumors) is not represented for it is easily recognized, its benignancy and short course known; thus, it is infrequently biopsied. Environmental and geographic factors may account for the higher incidence of malignant oral melanomas as compared with squamous cell carcinoma in America as reflected by our series and others,[104] in contrast with the reverse in England.[24, 27, 127]

This geographical difference requires further investigation.

Martin and Holland [70] collected 122 case reports of cancer occurring in the mouth, throat, and nasopharynx of animals and 35 unreported cases from zoos, veterinary schools, and veterinarians. In their series, cancer of the oral cavity was as common in the horse as in the dog. This finding was not demonstrated by a similar series collected by Schlumberger.[104] Wintzer,[126] in a series of 2,100 horses, found 44 tumors. In our series, collected from institutions over a period of many years, an overwhelming number of specimens are from dogs and only 20 oral neoplasms are from Equidae. Even after allowances are made for the relative population of horses and dogs and "endearment factor," it seems unlikely that oral tumors in the horse and dog should have similar percentage incidence in their respective populations.

On the other hand, Martin and Holland [70] found oral cancer to be about four times more common in dogs than cats. The relatively greater incidence of canine neoplasms has been pointed out by others.[24, 60, 73] This is even more marked in our series of oral neoplasms and may reflect a species difference. In the dog, squamous cell carcinoma of the tongue is rather rare, but in the cat this tumor has been said to be comparatively common.[25, 31] However, our series does not bear this out except in relation to the total number of feline tumors. This may reflect the inadequacy of our sampling of the cat population. As in man, the predominant site in the cat is the ventrolateral surface. In dogs, about 10 per cent of all neoplasms occur in the oral cavity.[28] A similar figure can be calculated for cats.[24, 25, 31]

Sex seems to play a role in the incidence of certain animal neoplasms but not in others. In the 1,000 general neoplasms collected by Mulligan [73] from 809 dogs, 407 were from males, 392 from females, and 10 from dogs of unknown sex. When these figures were corrected for the greater incidence of male dogs in the general population, it appeared that neoplasia was slightly more common in bitches. Comparable figures on general canine neoplasia were given by Cotchin.[28] In the cat, Cotchin [31] reported that of 356 tumor-bearing animals 253 were males, a proportion of 7:3. Since this is an unadjusted figure, conclusions can not be drawn. It is of interest that, in his series, all cats with squamous-cell carcinoma of the tongue were males.[25]

In a survey of 1,000 general neoplasms of the dog, Mulligan [73] found that about 45 per cent were malignant. A slightly higher incidence was experienced for oral neoplasms in our series; 157 (52%) of 304 canine oral tumors were malignant. In the cat, the percentage of malignancy is considerably higher. Cotchin [31] found that over 75 per cent of his series of feline tumors were so classified.

Generally, as in man, the incidence of neoplasms in other animals increases with age.[70, 73] Malignant tumors usually do not occur in younger animals.[21, 25, 120] Cohrs [21] found cancers in about 3 per cent of dogs necropsied when less than 10 years of age; in 25 per cent after 10 years of age. Cotchin [25] reported in 1952 than 68.5 per cent of cats with tumors were between 6 and 12 years old. Half of the malignant tumors in our series occurred in animals older than 10 years of age. A conversion table of equivalent age between man and dog was published in 1954.[61]

Benign Tumors

*Fibromatous Epulis of Periodontal Origin**.—The term "epulis" is used to designate fibromatous gingival tumors irrespective of their inflammatory or neoplastic nature. Clinically, they are hard, firm, usually sessile growths exhibiting a smooth or somewhat nodular surface. They may be localized or generalized. When generalized, they may involve the whole arch and simulate the "idiopathic" variety of fibromatosis seen in human beings. These growths may be at times so extensive as to cover the teeth entirely. Other investigators have called this lesion "fibromatosis gingivae" or merely "gingival hyperplasia."

Mulligan [73] and Riser [99] described gingival tumors in dogs and the latter stated that they were more common in Boxers and Boston Terriers. In a series of 226 tumors involving the mouth and nose, Riser reported that 150 were epulides. In 1952, Burstone, Bond, and Litt [19] described a familial gingival hypertrophy in Boxers of close ancestry. Microscopically, their lesions were characterized by central areas of mature lamellated bone and by nests and

* While this lesion should be included in the section "Tumors and Anomalies of Dental Origin and Dental Diseases," a special place has been devoted to its discussion because of its importance.

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cords of cells suggestive of odontogenic epithelium.

In the present series, 28 (53%) of 55 nonmalignant oral neoplasms in dogs were classified as epulides. The only epulis other than in dogs was in a Bengal tiger that was not included in this series. The microscopic appearance was identical with that seen in dogs. Cotchin [24] found 22 of 84 canine tumors to be epulides, corresponding to 96 of 379 canine oral lesions in the present series. The sex was known in 67 of our cases. When corrected for greater male population, there was no sex difference. The ages ranged from 2 to 14 years with an average of 7.5 years. Of the 90 known breeds, only 17 were Boxers. This figure was not corrected for breed frequency but it was the authors' impression that breed was not the sole determining factor.

Microscopically, 60 per cent of the growths exhibited calcified tissue which ranged from osteoid to mature lamellated bone. This bony tissue was invariably associated with a cell-rich bone-forming matrix. A cementum-like material was present in 3 cases. Inflammatory cells were observed only where the lesions were subjected to biting trauma. Epithelial proliferation similar to that observed by Burstone *et al.*[19] was seen in about 60 per cent of the epulides. There was high correlation between epithelial proliferation and osteoid or bone formation. Only rarely were cystic changes observed in the epulides, whereas Burstone *et al.*[19] cited such changes in 1 in 4. Our single case of "gingival cyst" was of considerable size, with a stratified squamous epithelial-lined cavity. The presence of bone in the lesion caused the authors to suspect that this was an exaggerated variation of the same lesion. *By virtue of the odontogenic epithelium, and the bone and the cementum that these lesions so frequently contain, it seems probable that this lesion is of periodontal membrane origin.* Because of the pseudoepitheliomatous hyperplasia and odontogenic epithelium they are frequently mistaken for ameloblastoma. In view of our findings it seems speculative to draw a parallel between a "familial multilocular cystic lesion" of man and the Boxer [19] (fig. 6 to 10).

Canine Oral Papillomatosis.—One of the more common types of benign oral neoplasms in the dog is viral papillomatosis. The human counterpart of this lesion is the dermal wart. Their histological features are similar and both are caused by viruses. A histologically similar infectious cutaneous papilloma occurs in the dog.[3]

The infectious nature of canine oral papillomatosis was described before the turn of the century. Findlay,[35] Goodlow,[38] DeMonbreun and Goodpasture [32] demonstrated the viral and immunological aspects of this disease. The virus is intracellular and is species and organ specific. It involves the oral mucosa, and occasionally the nasal mucous membranes of the dog. A lifetime immunity to reinfection is produced. The disease occurred most commonly in pups or young adult dogs, the mean age in this series being 1.0 year. There was no sex preference. The incubation period varied from 30 to 33 days. Although they may occur anywhere in the mouth, most often the lesions are seen about the inner margin of the upper lip. Other favorite sites are the lower lip, tongue, palate, and even the nose. They do not usually extend into the pharynx. They vary in size and shape from whitish, flat-

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Fig. 6—Canine fibromatous epulis of periodontal origin. Note numerous strands of odontogenic epithelium and lamellated bone.

Fig. 7—High power view of the epithelium shown in figure 6.

Fig. 8—Canine fibromatous epulis of periodontal origin. Note cell-rich bone forming matrix so common in these lesions.

Fig. 9—Canine fibromatous epulis of periodontal origin. Either bone or cementum may be found in this lesion. This, in addition to the odontogenic epithelium, points to its periodontal origin.

Fig. 10—Human fibromatous epulis of periodontal origin. Occasionally, the odontogenetic epithelium through metaplasia will proliferate and assume squamatoid characteristics. This has led, not uncommonly, to erroneous diagnosis of ameloblastoma or squamous cell carcinoma.

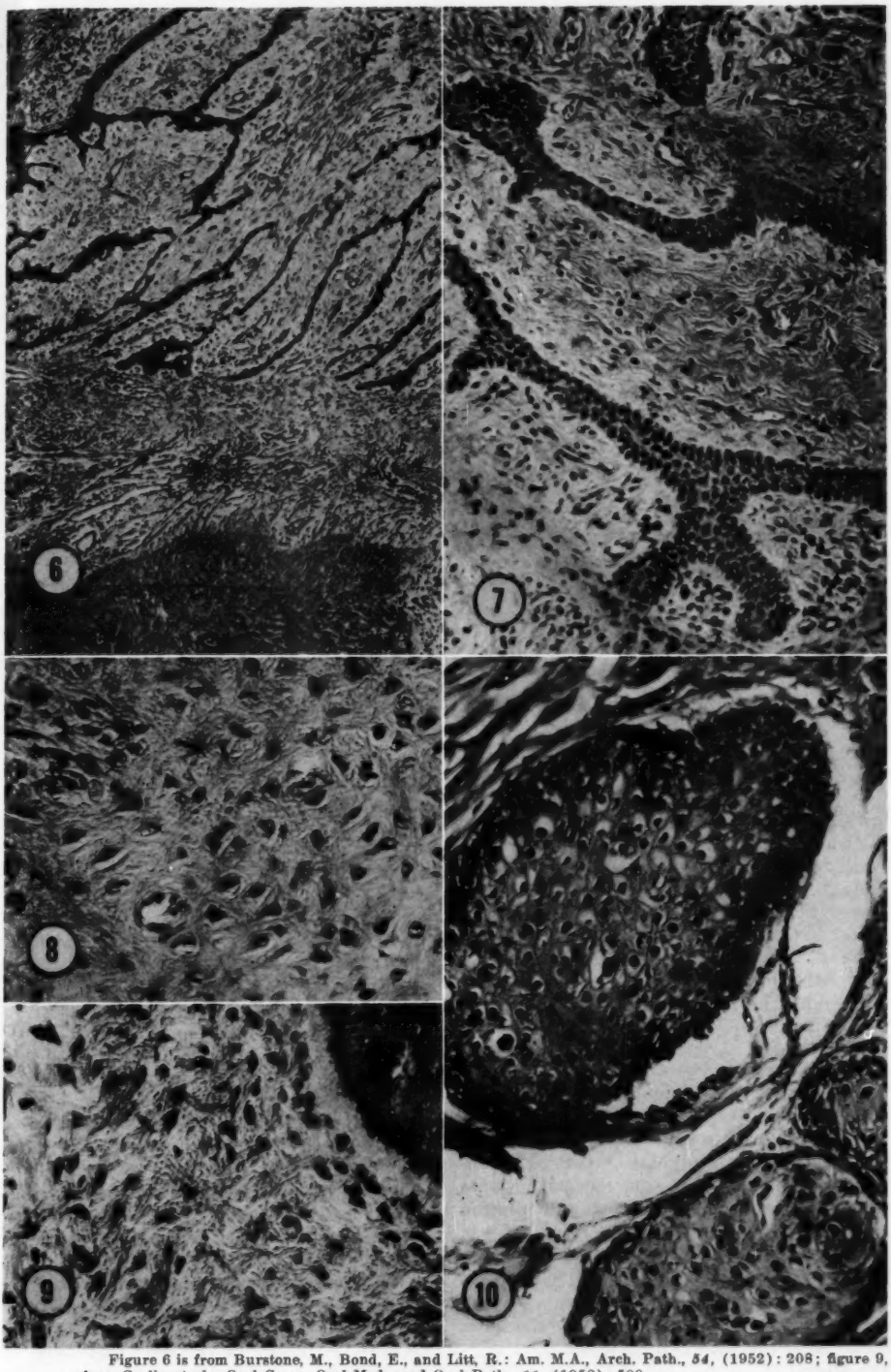


Figure 6 is from Burstone, M., Bond, E., and Litt, R.: Am. M.A., Arch. Path., *54*, (1952): 208; figure 9, from Gorlin *et al.*: Oral Surg., Oral Med., and Oral Path., *11*, (1958): 500.

XUM

tened, or smooth nodules a few millimeters in diameter, to grayish pedunculated, cauliflower-like growths. They regress in size within six weeks and become darker and shriveled and finally disappear within another week or two, leaving no scar.

There is marked similarity between the histological appearance of canine oral papillomatosis and than of human warts. The

TABLE 2—Benign Tumors of Domestic Animals

Tumors	Canine	Feline	Equine	Bovine
Fibromatous periodontal epulis	96	..	..	..
Oral papillomatosis, canine	24	..	..	..
Noncanine papillomata	..	..	2	..
Papillomatosis (x disease)	..	..	..	15
Fibroma, neurofibroma, fibroxanthoma	11	1	2 mules	..
Hemangiopericytoma	2	..	..	..
Lipoma	1	..	..	1
Osteoma	..	..	2	..
Chondroma	3	..	..	..
Adenoma				
Sebaceous differentiation	2	..	..	..
Mucous salivary gland	1	..	..	..
Fibroadenoma	1	..	1	..
Nevus (pigmented and neuro-)	5	..	..	..
Total	146	1	5 horses 2 mules	16

initial change is a simple hyperplasia of the epithelium. As acanthosis increases, small islands of prickle cells seem to lose their intercellular bridges. This is followed by an increase in the thickness of the cornified layer. The cells of the basal layer remain normal in size but the cells of the outer stratum spinosum increase greatly in size and exhibit intensely stained nuclei. With this epithelial proliferation, the surface is thrown up in folds containing connective tissue cores. In older lesions, keratohyaline-like eosinophilic cytoplasmic and basophilic nuclear inclusions are seen. There is frequently an abundance of mitotic figures in the malpighian layer. The corium is usually richly vascular with variable numbers of lymphocytes and plasma cells (fig. 11 to 13).

Among 170 benign oral lesions (table 2), 24 were examples of infectious papillomatosis and 2 of noncanine papillomata. It is easy to separate these on microscopic basis alone, for the characteristic swollen cells are absent.

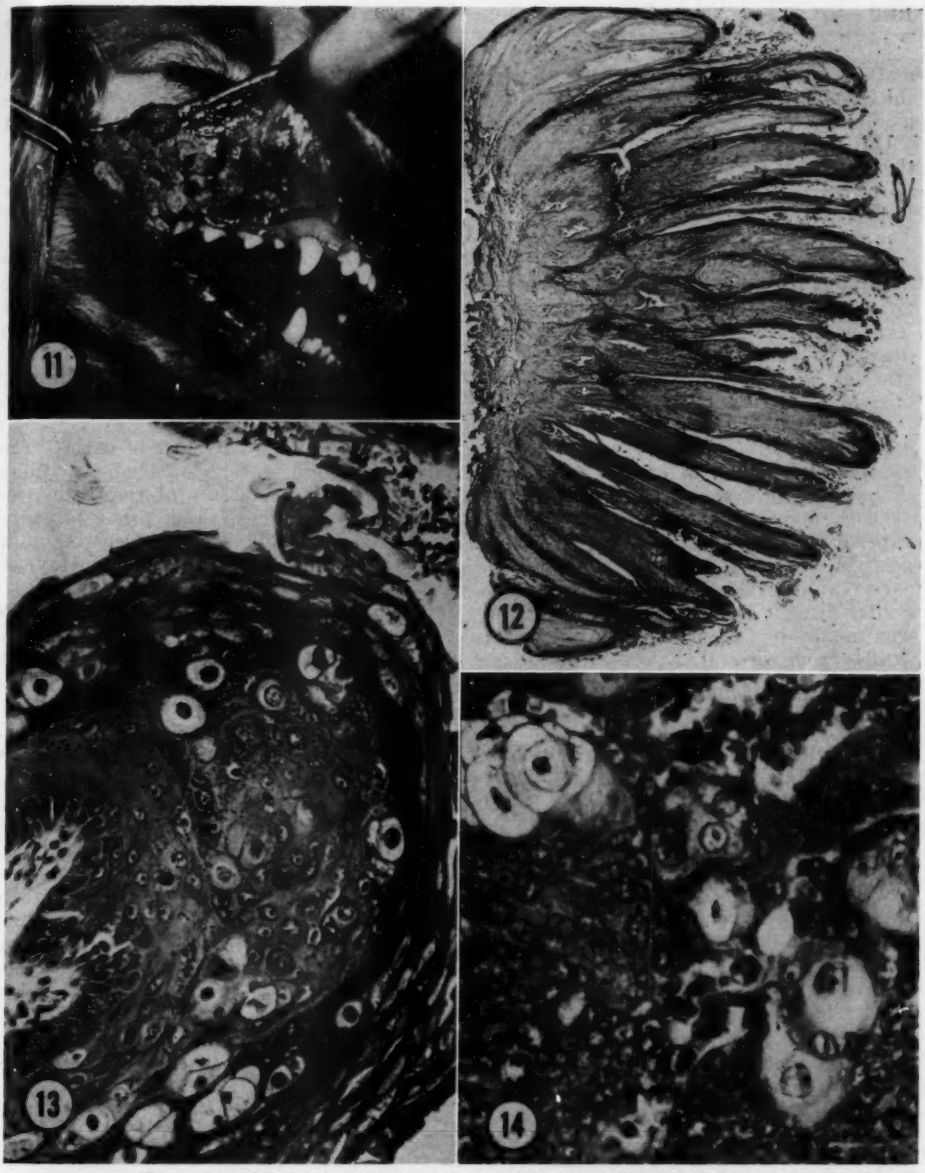
Fibroma, Neurofibroma, Fibroxanthoma.—Several canine gingival tumors (technically, epulides) that did not conform microscopically to the criteria of fibromatous periodontal epulis have been placed under the above heading. Ten were in dogs, 2 in mules, and 1 in a cat. Of special difficulty is the separation of a lesion seen in mules and horses than has been called "equine sarcoid." It is not unlikely that the 2 cases in mules belong in that category. This rather unique condition is discussed in another section.

Lipoma.—Only 2 lipomas were observed, 1 in a dog and 1 in a cow. The microscopic picture was identical with the same lesion in man.

Sebaceous Adenoma.—Although sebaceous adenoma is reported commonly in dogs, there seems to be a marked difference in the rate of reported incidence. Mulligan [73] found 45 "sebaceous adenomas" and 15 sebaceous gland carcinomas in a series of 1,000 tumors. All of these apparently occurred in the skin. He indicated a higher incidence in male dogs. Willis [125] recorded 4 "sebaceous adenoma of skin" in 204 specimens. Cotchin [27,28] observed 14 in 422 skin tumors or in 1,150 tumors of all systems in the dog. In his discussion of cat neoplasms, however, sebaceous adenoma was not seen.[25,31]

Perhaps the differences in incidence reflect differences in nomenclature and interpretation. The lesions that Mulligan [73] illustrated as sebaceous adenoma (his Plate XXIII fig. C, D) might be interpreted by some as sebaceous hyperplasia, while some might interpret his sebaceous gland carcinomas (Plate XXIV, fig. B, C) as either sebaceous adenomas or basal cell carcinomas. All degrees of gradation exist in the dog. The transition from well-differentiated lobules to basaloid areas (Plate XXIV, fig. B) is not uncommon. Clinical evidence supports the interpretation that well-differentiated lesions are benign neoplasms, and they are commonly designated as "adenoma" in veterinary literature. The lesions composed predominantly of basaloid reserve cells are usually locally aggressive only and thus their designation as carcinomas is frequently questioned.

The usually well-circumscribed nonencapsulated tumor is separated into numerous lobules by bands of collagenous connective tissue. The basic cell type is basaloid with maturational transition to



Figures 11 and 12 are from Gorlin *et al.*: Oral Surg., Oral Med., and Oral Path., *11*, (1958): 500.

Fig. 11—Canine oral papillomatosis. Note numerous wartlike growths on the buccal and labial mucosa of this young Golden Retriever.

Fig. 12—Canine oral papillomatosis. This self-limiting, short-lived virus disease produces papillomatous growths that superficially resemble the arborescent papillomas frequently observed in the tonsillar and uvular areas in man.

Fig. 13—Canine oral papillomatosis. High power photomicrograph of one of the finger-like projections showing swollen epithelial cells with intracytoplasmic inclusions.

Fig. 14—Canine sebaceous adenoma. High power photomicrograph demonstrating germinative and mature sebaceous cells, as well as intermediate forms.

XUM

mature sebaceous cells, the percentage of the two forms varying from lobule to lobule.

Our 2 examples of this tumor, both from the mucocutaneous junction of the lips, were in a 9-year-old German Shepherd Dog [40] and an 11-year-old Cocker Spaniel. The origin was inferred to be in the skin surface, although the mucosa was involved in both cases. One was called a sebaceous adenoma; the other an adnexal tumor with sebaceous differentiation due to its greater degree of anaplasia. For ease of consideration, we have grouped both together (fig. 14).

Tumors and Anomalies of Dental Origin and Dental Diseases.—Colyer's [23] comprehensive early study of comparative dental pathology surveys anomalies of dentition of both domesticated and wild mammals with regard to variation in number, shape, position, eruption, overgrowth, hypoplasia, caries and its sequelae, abrasion, periodontal disease, and odontomes. The study was made by examination of prepared skulls from several European museums. Certainly this is a neglected area of pathology and merits considerable further investigation.

Supernumerary Teeth.—Supernumerary teeth are fairly common in dogs. It has been estimated that about 9 per cent of dogs, regardless of breed, will have extra teeth. Bulldogs and Spaniels not uncommonly have supernumerary incisors while Greyhounds have a tendency toward extra premolars. Less commonly, extra teeth are seen in the horse (occasionally maxillary premolars, the so-called "wolf teeth") while in the bovine, ovine, and porcine species they are exceedingly rare.

Aplasia of Teeth.—Congenital absence of teeth is uncommon. Colyer [23] indicates, however, that the Husky dog occasionally has a missing first premolar.

Fusion, Gemination, and Concrescence.—These three conditions are rare but gemination has been reported in the incisors of a horse. Hypoplastic enamel is seen in animal rickets, and in fluorosis in cattle, sheep, and horses. Abrasion of teeth is commonly seen in animals, such as horses and cows, that chew coarse feed.

Caries.—Dental decay is common in the horse. It has been estimated that about 12 per cent of horses will have evidence of dental decay. It is more pronounced in older animals, and seems to have a predilection for the occlusal surfaces of maxillary first molars. This may be due to a structural defect.

The cat is unusually free of caries. The dog, although rarely affected, at times has the occlusal surface of the distal molars involved. Sheep seem prone to cervical caries. Possibly this is also due to a structural defect and seems worthy of investigation.

Caries, once gaining a foothold, proceeds much as it does in man. However, in species such as horses, cattle, and swine, periapical involvement with massive maxillary destruction may occur, due to their unusually long teeth. If maxillary teeth are involved, a severe maxillary sinusitis may result. This is especially common in the horse.

Periodontal Disease.—Periodontal disease is not as widespread in animals as it is in man. Of all domesticated species, dogs, cats, and horses are the most susceptible. Some breeds of dogs are more prone than others, due to a peculiar jaw relationship. This is especially true in the case of the Bulldog. The inbred mandibular prognathism makes for exposure of the lower incisors, which are especially prone to periodontoclasia. That this may be due to failure of cleansing with the lower lip is possible. The Bulldog also seems prone to involvement of the maxillary second and

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Fig. 15—Bovine ameloblastic odontoma. This developmental anomaly consists of both hard and soft tissue. Dentin and dentinoid may be seen in this photomicrograph.

Fig. 16—Bovine ameloblastic odontoma. In another area of the same tumor several islands of odontogenic epithelium in a loose mesenchymal stroma are observed. Note the ameloblastic cells, the polarity of the nuclei, etc.

Fig. 17—Bovine hyperkeratosis. Note numerous raised hyperkeratotic plaques on dorsum and lateral surfaces of tongue.

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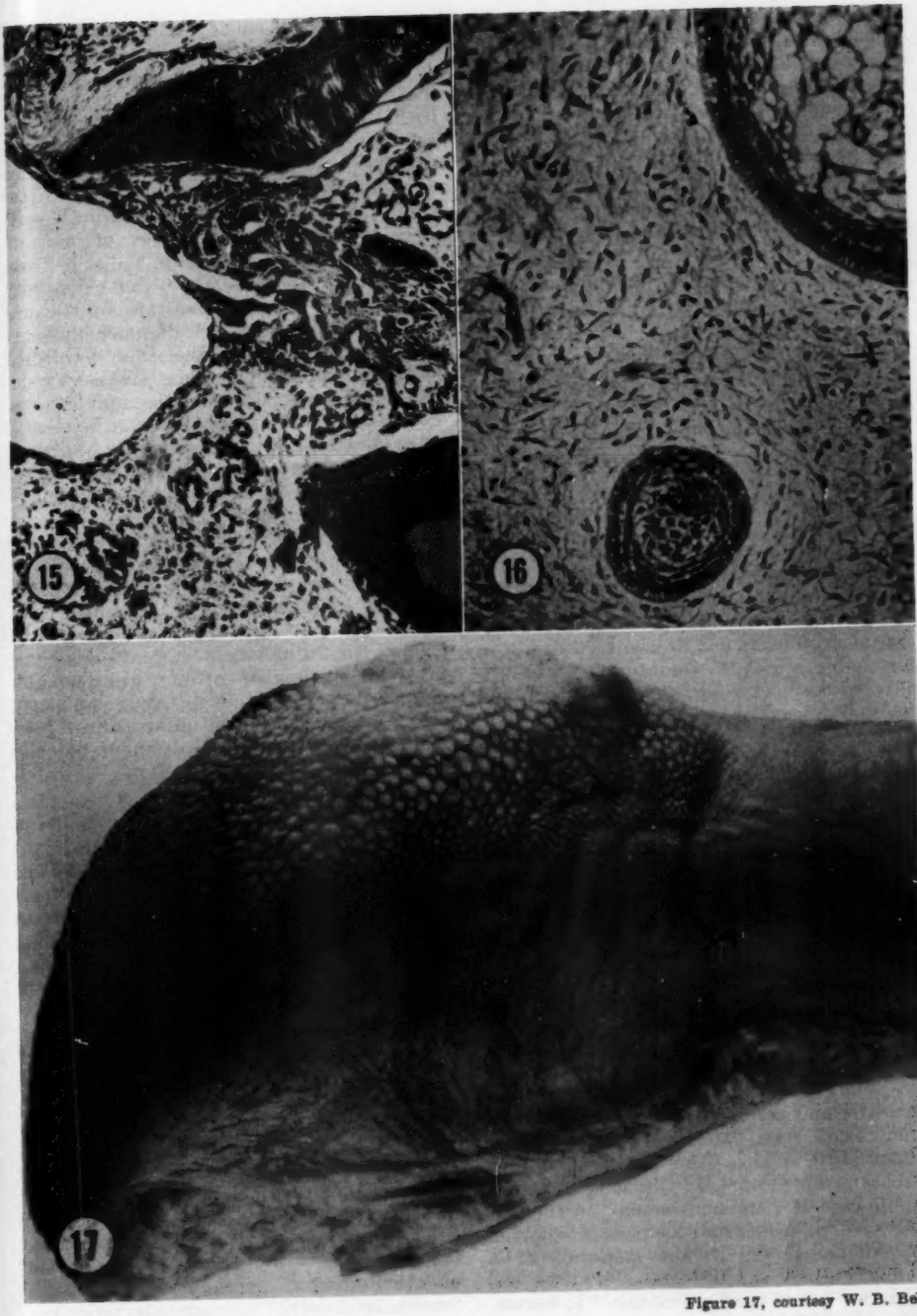

Figure 17, courtesy W. B. Bell.

third premolars which, lying oblique to the line of the arch, are disposed to food impaction. Periodontal disease is less commonly observed in the cat. Occasionally, the maxillary fourth premolar is involved.

The horse is probably the most susceptible domesticated animal. Colyer [23] indicated that about one third of horse skulls examined exhibited periodontal disease, especially in the mandible, due to food impaction of awns, grains, and the like.

Cysts and Tumors of Odontogenic Origin.—Cysts of dental origin, either periapical or dentigerous, are rare in animals. Dentigerous cysts have been reported in horses, pigs, and sheep. They seem to be most common in the last species in the region of the mandibular incisors or canine teeth.

Tumors of dental origin in animals are not common. In his survey of 1,000 canine tumors, Mulligan [73] listed 3 odontogenic tumors which he apparently interpreted as adamantinoma. However, he gave inadequate details to permit evaluation. Cotchin,[27] in 1,150 canine specimens, found no dental neoplasms nor did he record any in his feline series.[25] Odontomas were discovered in horses by Bland-Sutton [10] and Schlumberger.[104] Colyer[23] observed complex composite lesions in the molar regions of a sheep and an ox and a compound composite odontoma in the cuspid area of a dog. No report was found of soft odontomas (odontogenic myxoma, odontogenic fibroma, ameloblastic fibroma) in animals. Cementoma is not an uncommon lesion in the horse, occurring in the molar or premolar region. It is apparently rare in other animals.

The early reports of ameloblastoma as "adamantinoma" are of European origin. They have been reviewed by Hoogland [49] and Schmal.[105] Most cases were reported in cattle, especially in the mandibular incisor region; Fölger [37] reported three cases in cattle and referred to two in cats. This tumor has been described in the rat by Ratcliffe [98] and in the cat and ox by Nieberle and Cohrs.[77] Olafson [81] cited a single case of "adamantinoma" in the former species. Jasper and Kanegis [53] reported an adamantinoma in the mandible of a young bull. Cotchin [29] states that he has seen two "true adamantinomas" in horses. Zegarelli [130] has indicated the occurrence of "adamantoblastomas" in a select tumor-prone stock of mice; and Bullock and Curtis [18] have discussed odontomas in rats.

Examination and re-evaluation of all animal gingival tumors labeled "ameloblastoma" or "adamantinoma" at the A.F.I.P. revealed that all should be considered peculiar variations of the fibromatous periodontal epulis. Odontogenic epithelial rests may range from the typical rests of Malassez to broad sheets of mature-appearing cells arranged in palisade fashion along the borders. The cellular diversity has possibly been responsible for several reports in the literature of ameloblastoma or adamantinoma. Whether these published cases were all true ameloblastomas or ameloblastic odontomas or variations of fibromatous periodontal epulis could not be clearly ascertained. Published evidence indicates that several cases, with possible exception of cases cited by Olafson [81] and Fölger,[37] should be classified as the latter.

We observed only two examples of "tumor" of dental origin—an ameloblastic odontoma [8] in the mandible of a 2-year-old Holstein-Friesian bull and a complex composite odontoma in a dog. As in man, the tumors consist of two or more components: nests or strands of proliferating odontogenic epithelium and a hard connective tissue resembling cementum or dentin. Normal tooth structural arrangement was not observed (fig. 15, 16).

Hemangiopericytoma.—Our 2 examples of hemangiopericytoma were in the lip of a 6-year-old female and in the chin of an 11-year-old male, both Cocker Spaniels. The former has been reported by Gorlin and Chaudhry.[41] Mulligan,[75] as recently as 1955, described 68 cases in dogs, but none were in the oral region. Stout and Murray [117] originally described this tumor in 1942 and the former published several excellent reviews.[76, 114, 116] In a recent paper, Stout indicated that he had 12 examples of this tumor in the oral cavity of man (tongue, lips, and palate—each 3; and alveolar process, parotid, and cheek—each 1).[115] We are unaware of any report of this tumor other than in man and dog except for one case in a cow recorded by Yost and Jones.[129]

The tumor consists of huge numbers of capillary-like structures lined by normal endothelium but surrounded by packed "epithelioid cells," presumably representing the cells originally described by Zimmerman as "pericytes." The determina-

tion of the exact position of these cells in relation to the reticulin sheath can not be well determined with routine hematoxylin and eosin stain. A silver reticulin stain is employed to differentiate this tumor from those of vascular endothelium, whose cells proliferate within the reticulin sheath.

Oral Papillomas Associated with Bovine Hyperkeratosis.—Bovine hyperkeratosis (x disease) was originally described by Olafson [82] in 1947. Initially, its cause was unknown [82, 86, 87] but later it was found to be due to highly chlorinated naphthalene compounds.[4-7, 44-46, 48, 83, 106, 109, 123] In cattle,[46, 82, 83] it is characterized by erythematous erosive areas on the lips, tongue, hard palate, muzzle, and gingivae. These oral lesions gradually change into papillomatous or warty growths. Intoxication by the same compounds has been induced in several species,[106] but only in cattle have oral papillomas been associated with the disease.

Recent investigations implicate a virus in the production of the papillomatous oral lesions, which may develop in association with hypovitaminosis A alone.

In our 15 cases, the only oral lesions available for study are papillomas. They differ from the lesion of canine viral papillomatosis by being simply focal hyperkeratosis, without the swollen cells so characteristic of the canine lesion. Metaplasia of salivary gland ductal epithelium to stratified squamous type occurs in this disease, also, but no sections were available for study in our cases (fig. 17).

Osteoma.—Schlumberger [104] cited Cole as finding an osteoma and osteofibroma in the maxilla of a dog. In view of what we know about the frequency of bone in canine epulides, it is possible that these tumors were examples of this latter entity. Feldman [34] reported several osteomas and osteofibromas in the jaws of horses and cows. The tumor in horses consisted of mature bone with a minimum of fibrous connective tissue stroma. A similar tumor has been described by Fisher [36] (fig. 18).

MALIGNANT TUMORS

Melanoma.—Melanoma was the most common malignant tumor of our series. One was in a mule; the others were in dogs. Whether this is in some way related to the melanotic pigmentation so often seen in the oral mucous membrane of dogs, as Schlumberger [104] contends, is not known; but in our series, the oral tumor was much more common in dark-colored dogs. Cotchin [25] observed no oral melanomas in a large series of cats, while Cole [22] cited but two cases in cats. The horse, although frequently subject to melanoma, does not commonly have primary lesions of the oral cavity.[72, 104]

TABLE 3—Malignant Tumors of Domestic Animals

Tumors	Canine	Feline	Equine	Other
Melanoma	62	..	1 mule	..
Squamous carcinoma	35	8	4 horses	1 bovine
			1 mule	1 ovine
Lymphoepithelioma	2	..	..	..
Basal cell carcinoma	7	..	..	..
Salivary gland carcinoma				
Adenocarcinoma	3	..	..	1 ovine
Other	5	1	1 horse	..
Sarcoma				
Fibrosarcoma	11	..	2 mules	..
Chondrosarcoma	3	..	4 horses	1 bovine
Osteosarcoma	3	..	..	..
Rhabdomyosarcoma	1	..	..	..
Alveolar soft part sarcoma	..	1	..	..
Mast cell sarcoma	10	..	..	..
Malignant lymphoma	6	..	..	..
Transmissible venereal tumor	5	..	..	..
Myelocytoma	2	..	..	..
Total	155	10	9 horses	2 bovine
			4 mules	2 ovine

One of the most preplexing problems is the apparent difference of this tumor's incidence in England and in America. While melanoma appears to be the most common canine oral malignancy in this country, with squamous cell carcinoma a poor second,[22] the reverse seems to be true in Britain.[29] Withers [127] and Cotchin [27, 28] found that squamous epithelioma of the tonsil is overwhelmingly more common than any other oral malignancy. Whether this is due to an environmental factor is yet to be determined. In a rather large series of oral tumors, Riser [100] found an almost equal incidence of malignant melanoma and squamous cell carcinoma in the oral cavity of dogs.

Melanoma does seem to be more common in certain breeds. Mulligan [73, 74] found that Airdales, Scottish and Boston Terriers, and English Springer Spaniels were most frequently affected. He believed, as did Cotchin,[28, 29] that dark-skinned dogs were more prone to have this tumor. Our series of oral melanomas, although the breed data were missing in part, indicated that the disease was far more common in black dogs, especially the black Cocker Spaniel. The German Shepherd Dog rated a poor second.

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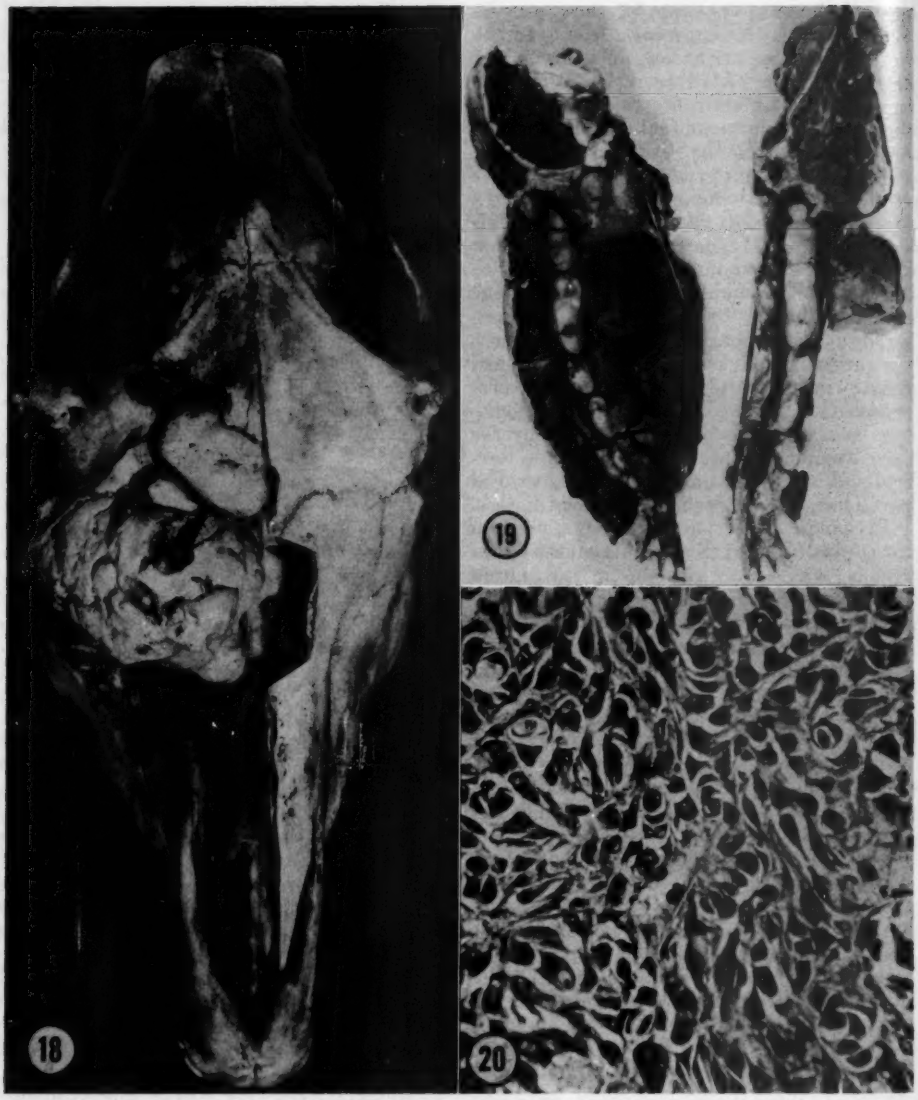

Figure 18, courtesy Dr. A. K. Fisher, University of Iowa School of Dentistry.

Fig. 18—Equine osteoma. Tumor mass of dense bony tissue occupying maxillary sinus area of horse.

Fig. 19—Canine malignant melanoma. Tumor arose in floor of mouth and invaded mandible, especially about the teeth. This photograph contrasts the involved and normal sides.

Fig. 20—Canine malignant melanoma. Note pleomorphism of cells, tendency toward alveolar arrangement. This tumor demonstrated only sparse pigment.

Sex seemed to play a significant role; of our 47 cases in which sex was ascertained, 33 were males, 11 females, and 3 spayed females.

Age seems to play a significant role also, for 26 (75%) of Mulligan's cases [73] occurred in dogs between 9 and 14 years of age. In our series of 51 dogs with ascertainable ages, the mean was 11.5 years and about 75 per cent were over 9 years old.

The lips and gums were the most common sites of origin though the palate, buccal mucosa. and floor of the mouth were also involved. The rarity of tongue involvement in the dog may be significant. In fact, involvement of the canine tongue with any lesion, save that of infectious papillomatosis, is exceedingly rare. Cotchin [27] reported two carcinomas and 1 sarcoma of the tongue in his series of canine neoplasms, and McClelland [68] cited a malignant melanoma of the tongue of an 8-year-old male Chow.

Melanoma has been experimentally produced in dogs.[92] We observed 3 cases of melanoma of the canine tongue. All were on the dorsum at the base. Melanomas usually are somewhat firm, gravish or brownish black tumors that rapidly enlarge and break down, often developing marked ulceration, hemorrhage, and metastasis within weeks or months. The lungs seem to be a common site of metastasis.

Microscopically, canine melanomas have a wide range of histological patterns. Usually, the new growth is made up of large epithelial cells supported, and separated into indistinct lobules, by bands of collagenic connective tissue. The epithelial cells, arranged in sheets, nests, whorls, or alveolae, are frequently polymorphous with a single nucleolus-containing nucleus. Multinucleated forms are occasionally seen. Some single cells will exhibit fine melanin granules in the cytoplasm while others are completely amelanotic. Mitotic figures are usually numerous. Areas of necrosis and inflammation are common to most large tumors. All of these observations also apply to the metastases.

Cotchin [28] classified them as the malignant melanoma, the benign cellular melanoma, and the benign fibrous melanoma. The first two types seem to correspond to Mulligan's "cancerous" and "noncancerous" types.[73] Cotchin's third type seems to have been described by Spurrell [112] as "spottily-pigmented neurofibromas," which he considered to be neurofibromas that

TABLE 4—Differential Diagnosis of Tumors of Domestic Animals

	Transmissible venereal tumor	Mast cell tumor	"Myelocytoma" of the skin	Malignant lymphoma
Species	Canine	Canine Feline Bovine	Canine	All species, principally bovine and canine
Usual sites involved	Usually genital	Usually hindquarters	Skin	Lymphoid tissue throughout body
Microscopic characteristics 1) Cell type	Cell about size of lymphoblast, but nuclear membranes and chromatin stain more deeply.	More cytoplasm than lymphocyte and darker staining chromatin.	Resemble immature cells of myeloid series.	Lymphocytic, lymphoblastic, or, rarely, reticulocytic.
2) Mitotic activity	Large number of mitotic figures.	Few mitotic figures.	Numerous mitotic figures.	Variable mitotic activity.
3) Other features	Cellular and nuclear pleomorphism.	Cytoplasmic granules, more pleomorphism than T.V.T., darker staining nuclei.	Mature neutrophils, eosinophils commonly present, multinucleated and bizarre forms common.	Usually little pleomorphism, monotony is the general rule.
4) Giemsa and toluidine blue	Negative.	Positive metachromasia.	Negative.	Negative.
Clinical behavior	Low metastatic and infiltrative power.	Low metastatic but high infiltrative power.	Low metastatic but high infiltrative power.	Moderate infiltrative and metastatic power.

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were secondarily pigmented by a "sinking-in" of pigment cells from overlying epithelium.

Although all melanomas may be malignant, many seem to have a markedly benign course. To separate them into benign and malignant melanomas the following microscopic criteria were used:

Characteristics	Benign	Malignant
Arrangement of cells	Compact	Loose
Pigmented dendritic forms	Common	Rare
Nucleoli	Single	Two or more commonly seen
Areas resembling nerve sheath tumors	Present	Absent
Nuclear pleomorphism	Absent	Present
Bizarre mitoses	Absent	Present

Benign or noncancerous melanomas are characterized by cells of several types. They may be polyhedral or spindle-shaped. Many cells have dendritic processes so heavily laden with pigment that the nucleus can not be seen. The cells are usually compactly arranged and the spindle-shaped cells may take a bundle or palisade pattern somewhat resembling Meissner's corpuscles. The polyhedral cells are usually uniform in appearance and have an acidophilic cytoplasm. The nucleus is nearly always single and bears but a single nucleolus. Mitoses are not common and bizarre forms are not seen. A favorite site seems to be the eyelid [74] (fig. 23, 24).

In contrast, the malignant variety almost never exhibits dendritic forms. The cells are usually loosely arranged and rarely pigmented to the marked degree seen in the benign variety. On the other hand, amelanotic areas are commonly observed. Mitotic activity is great and bizarre forms are common. Nuclei are frequently large in relation to the amount of cytoplasm, and two or more nucleoli are common (fig. 19–22).

Both Mulligan [73] and Cotchin [28] believed that the majority of skin melanomas are benign. Exactly the opposite is true for oral melanomas. All but one in our oral series (and that came from the mucocutaneous portion of the lip) were of the "cancerous type." In contrast to the generally stated absence of junctional changes in canine melanomas,[58] about 20 per cent of oral melanomas examined by the authors revealed junctional changes adjacent to the tumor. This has also been the experience of Jones.[55] Perhaps if serial sections were obtained, a far higher percentage might have been detected. Because the oral melanoma is so commonly amelanotic and the cells so pleomorphic, a good philosophy is "in case of doubt, consider melanoma."

Squamous Cell Carcinoma.— Although squamous cell epithelioma behaves clinically in dogs and cats much as it does in man, its incidence appears to be considerably less.[22] In man, squamous cell carcinoma comprises over 90 per cent of oral malignancies, the lip and tongue being the most common sites.[8]

In the United States, available statistics do not bear out the reports of Withers [127] and Cotchin [27] in England that squamous cell carcinoma of the tonsil is overwhelmingly the most common canine oral malignancy. This may reflect a difference in geographic incidence, but large surveys in various centers in several countries would be necessary to establish this.

Martin and Holland,[70] having collected 122 case reports of oral and nasopharyngeal neoplasms from the literature and an additional 35 cases from zoos, veterinary schools, and veterinarians, presented an interesting comparative topographical incidence breakdown. They were presented with the same problems that we en-

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Fig. 21—Canine malignant melanoma. Note large numbers of pigment-containing melanoblasts. Pleomorphism is marked.

Fig. 22—Canine malignant melanoma. In contrast to the common belief, junctional change is not uncommon. The photomicrograph was taken of epithelium adjacent to frank melanoma.

Fig. 23—Canine "benign melanoma." Note extremely dense deposits of melanin in cells to the degree that cellular detail can not be visualized. There is doubt that these represent true melanomas.

Fig. 24—Canine "benign melanoma." Specimen seen in figure 28, after bleaching. Note nature of cells. These do not resemble either melanoblasts or nevus cells. This lends weight to the idea that this is not a melanoma.

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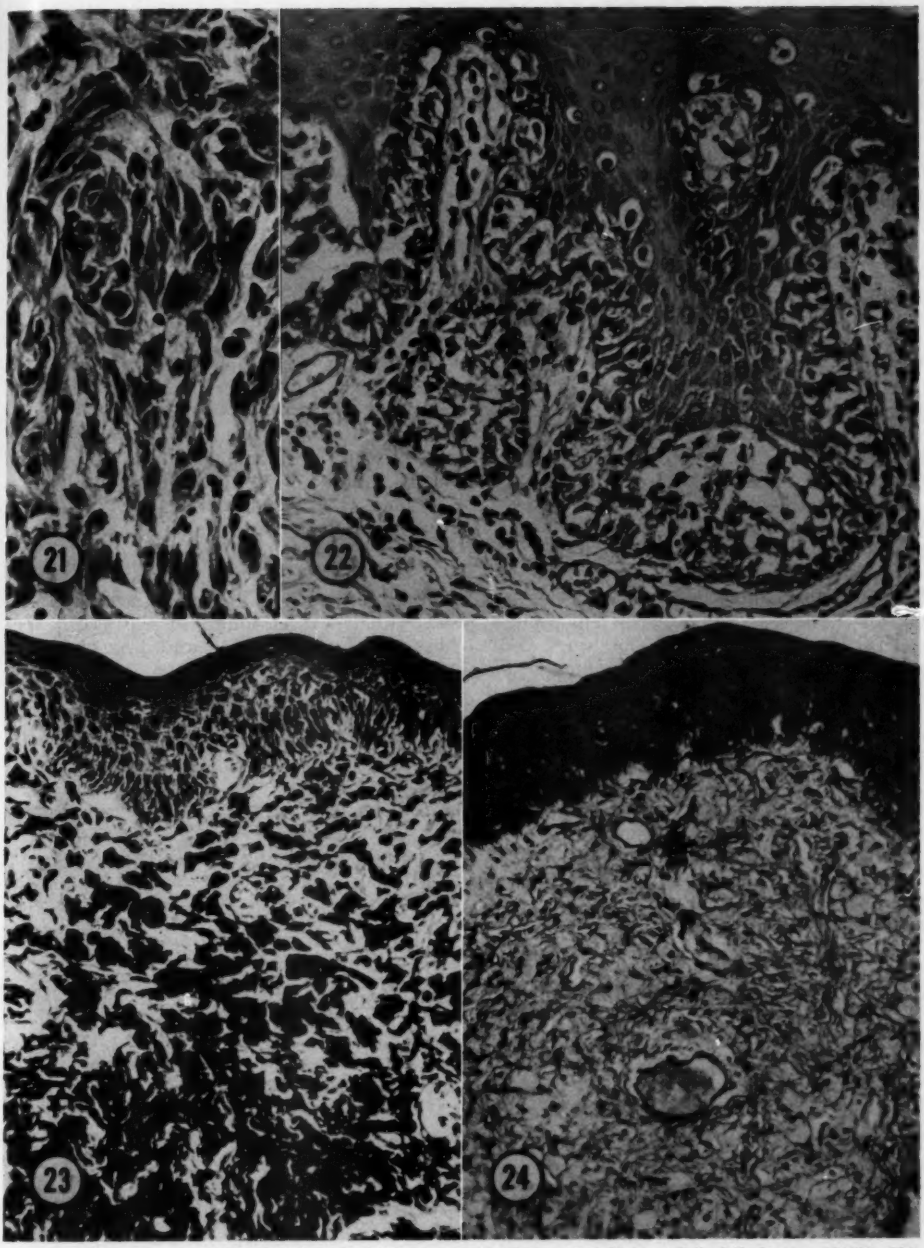

Figure 22 is from Gorlin *et al.*: Oral Surg., Oral Med., and Oral Path., *11*, (1958): 500.

countered; *i.e.*, nonspecification of exact location so characteristic of submitted material. Because of this, the inclusion of data on other than domesticated animals, and use of the general term "cancer" rather than more specific nomenclature, their figures are of little value. However, they did find that "jaw cancer in animals" is about four times more common than in man and that tongue cancer is about 1.5 times as common in man as in animals. They believe Power's [97] comments on the rarity of lingual cancer in animals to be somewhat exaggerated. In their grouped series, it is of interest that "cancer of the tonsil" comprises only 5 per cent of oral cancer.[70]

Another unusual phenomenon is the apparent absence in animals of man's precancerous oral lesion, leukoplakia. Our series contained 15 cases of hyperkeratosis. Since no dyskeratosis was present, we did not feel justified in calling it leukoplakia. We found no reports of leukoplakia in animals. As discussed earlier, hyperkeratotic lesions occurring in cattle as part of a disease complex called "bovine hyperkeratosis" or "x disease," is not true leukoplakia.

This series, and that of Cotchin,[29] indicate that the tongue is not commonly affected in the dog. In cats, squamous cell carcinoma of the tongue is common.[25, 31] Feldman [34] believed it to be the most common cat malignancy. That this is due to preening or fur-licking is unlikely, for lingual cancer does not appear to be common among other feline animals exhibiting this habit; [70] however, the population may be too small for a significant sample. The predominant site in the cat is the left ventrolateral surface at about the level of the frenulum.[25, 31] Deep invasion of the tongue musculature can occur, with early metastasis to local lymph nodes. The average age of the affected cats in our series was 10.5 years, the range being 3.5 to 16.0 years.

Tonsillar carcinoma was by far the most important oral malignancy in Cotchin's dog series. However, our series demonstrated only 8 cases in dogs. In the cat, tonsillar carcinoma is of less importance than lingual carcinoma.

There were 53 cases of squamous cell epithelioma in our series: 37 in dogs, 9 in cats, 4 in horses, and 1 each in a mule, a cow, and a sheep. One odd case was associated with papillomatosis and osteoid deposits. We observed no difference with regard to sex or breed. Terriers and Spaniels were more commonly affected, but the degree was not statistically significant.

There is no difference in microscopic appearance of this tumor in animals and in man (fig. 25, 26). The authors believe that undifferentiated squamous cell carcinoma is somewhat more common in animals than in man. Although diagnosed as lymphoepithelioma, this tumor was grouped with the broader category of squamous carcinoma for ease of handling.

Basal Cell Carcinoma.—Basal cell carcinoma in animals, as in man, is not an intraoral tumor. It is found in the skin. We observed 7, 6 in the lip and 1 in the submaxillary area, all in dogs.

Riser [100] found 16 examples of this tumor in 613 canine skin neoplasms. Cotchin [24] observed 47 in his group of 510 neoplasms and later reported 210 "basal cell and glandular tumors" in 932 canine skin neoplasms.[28] In a series of 40 feline skin tumors of epithelial origin, 31 were of basal or glandular origin.[25] Males were at least five or six times more commonly affected than females.[47, 99] Our present series is too small to draw any valid conclusions concerning sex distribution.

Head,[47] in 1953, cited 51 of these tumors in his series of 422 skin tumors in the dog; Cotchin [27] reported 14 that appeared to be of sebaceous origin and 16 of sweat gland

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Fig. 25—Canine squamous cell carcinoma. Lower jaw showing ulcerated area involving the periodontal structures and mandibular alveolar bone of a dog.

Fig. 26—Canine squamous cell carcinoma. Note islands of neoplastic squamous epithelium; also both pearl formation and keratinization of individual cells.

Fig. 27—Canine basal cell carcinoma. This pattern, much like that seen in lesions in man, is uncommon in animals. Most basal cell carcinomas rise from skin appendages.

Fig. 28—Canine basal cell carcinoma. This is a more common pattern. Note resemblance of epithelial strands to eccrine sweat glands. As in man, this tumor is not found intraorally.

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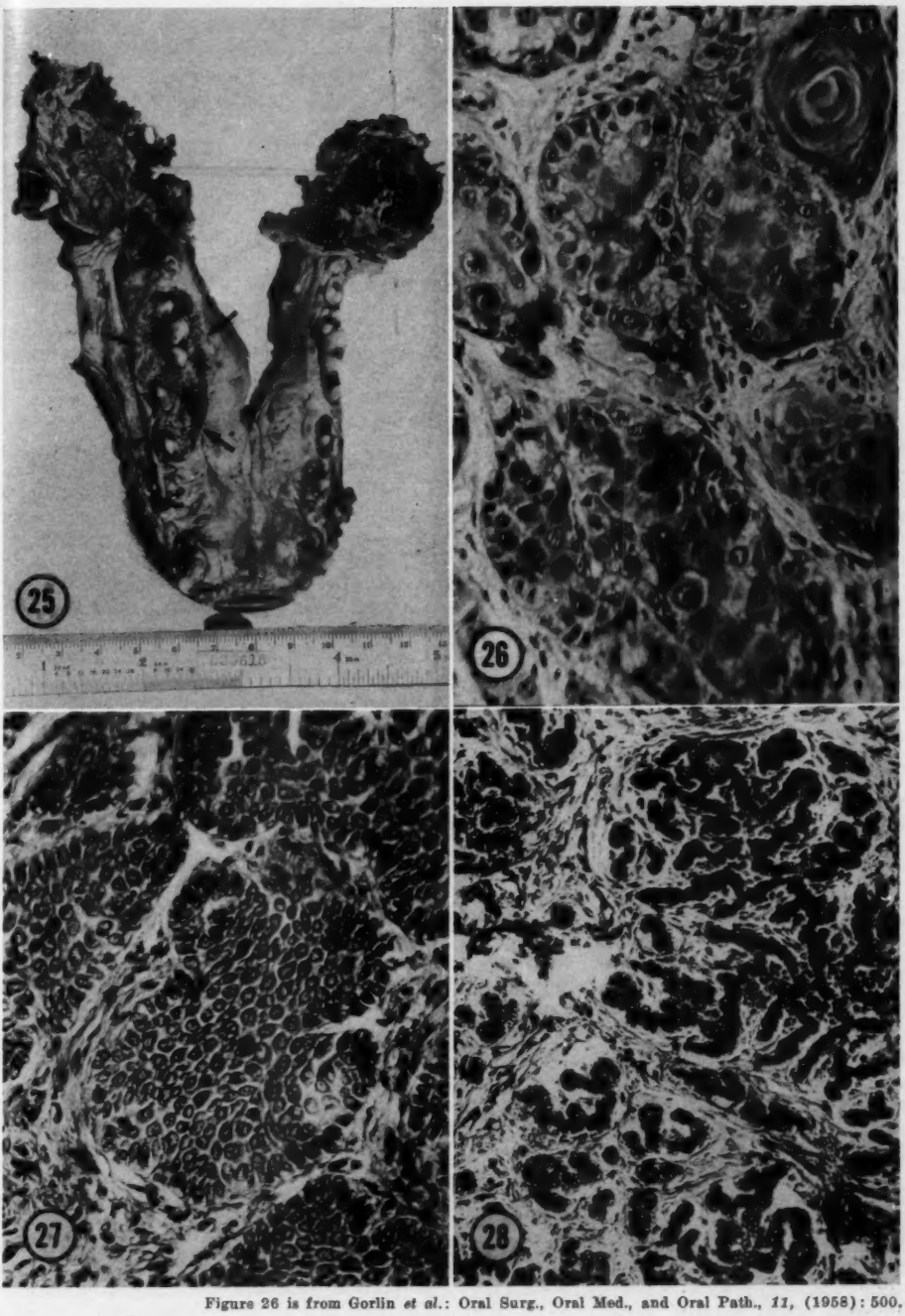

Figure 26 is from Gorlin *et al.*: Oral Surg., Oral Med., and Oral Path., *11*, (1958): 500.

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origin. The age varied from 10 months to 14 years with an average of 8 years. As in man, these tumors seen to enlarge slowly, ulcerate, and invade, but not to metastasize. Mulligan [73] has subdivided and subcategorized skin lesions much more than his English colleagues. He found 25 examples in his survey of 1,000 general canine neoplasms, 283 of these 1,000 being skin tumors of various types. Feldman [34] reported the existence of this tumor on the face, especially the jaws, ears, eyelids, and lips, although this point is disputed by Willis.[125] The origin may be in the epidermis, in glands, or in hair follicles. Willis believes that most basal cell carcinomas in dogs arise from sweat glands.

These tumors may resemble those commonly seen in man or they may exhibit an adenoid or reticular pattern, suggesting origin from sweat glands; or the tumor may consist of diffuse collections of closely packed uniform cells intermixed with pigment-laden cells reminiscent of noraml hair matrix. Mitotic activity is variable (fig. 27–29).

Mastocytoma.—The mast cell tumor, or mastocytoma, is chiefly a tumor of the skin. It was separated as an entity from other round cell neoplasms by Bloom [12] in 1942. The general and oral pathologist will find the older terminology somewhat confusing. For example, Feldman,[34] in 1932, in an attempt to simplify the myriad names applied to various round cell tumors grouped them into classes: *(a)* lymphomas, "benign tumors arising within lymph nodes, and other lymphoid tissues," and *(b)* lymphocytomas, "the malignant types of neoplastic hyperplasias of which the immature lymphocyte or its forerunner is the cell type." Mulligan [73] in 1949 divided canine round cell tumors into three categories: mast cell sarcoma, lymphosarcoma, and histiocytoma. Recently, malignant transformation of tissue mast cells with leukemia has been reported in man.[33] However, the clinical picture differed from that usually observed in the dog.

The mast cell tumor in the dog is frequently malignant. Although it may remain dormant for several months, or even years, it more frequently enlarges rapidly and may rarely metastasize widely.[78] Multiple lesions in the skin are more common. Because of its marked similarity to other round cell tumors, a special stain such as Giemsa's is employed to demonstrate the cytoplasmic granules which are not always evident in less differentiated, more clinically malignant, tumors. These granules, which have been shown to contain heparin, histamine, and serotonin, stain an intense violet with Giemsa's stain. Although there is still debate, the mast cell has been considered by most pathologists to be distinct from the basophilic leukocyte seen in human blood.[13, 84, 90] In spite of the high heparin content of these tumors, affected dogs have been shown to have normal bleeding and clotting times. We have had good results in staining with toluidine blue. Although this dye is not as permanent as Giemsa's, the staining technique is easily executed. Recently, a new dye technique for mast cells has been described [2] (fig. 30, 31).

We found 10 mastocytomas in this series; all in dogs. Six were in the upper lip, 2 in the gingiva or buccal mucosa or both, and 1 each on the hard palate and skin of the chin. Riser [100] found 3 in the nose and mouth of dogs. Mulligan [73] mentioned no intraoral mast cell tumors in his series although he indicated that occasionally they occur on the lip. Cotchin [24] found no oral tumors though he had 20 dermal examples in a series of 422 skin tumors; males seem to be more commonly affected. All but 1 in our series were males. Ages in various series, including ours, ranged from 2 to 15 years with an average of about 8 years. Both Cotchin's and Mulligan's series showed that Boxers, Bull Terriers, and Bulldogs were the breeds in which this tumor most commonly occurred. An excellent recent review of the status of the mast cell is that of Padawer.[89]

Malignant Lymphoma.—Among some species such as cattle and pigs and, to a lesser extent, dogs, cats, and horses, leukemic and nonleukemic lymphosarcoma are among the most common tumors. The latter term is used synonymously with "malignant lymphoma." In dogs, it occurs in all ages, though it may be slightly more common in the younger age group. More commonly, it is seen in the Scottish Terrier.[28, 73] There appear to be no sex preferences. The initial sign is frequently bilateral, symmetrical enlargement of submaxillary and cervical lymph nodes. Cats have this neoplasm more commonly than dogs though lymphosarcoma of the intestine in this species seems to be the most common site.[79] Cervical adenopathy in the cat is unusual.

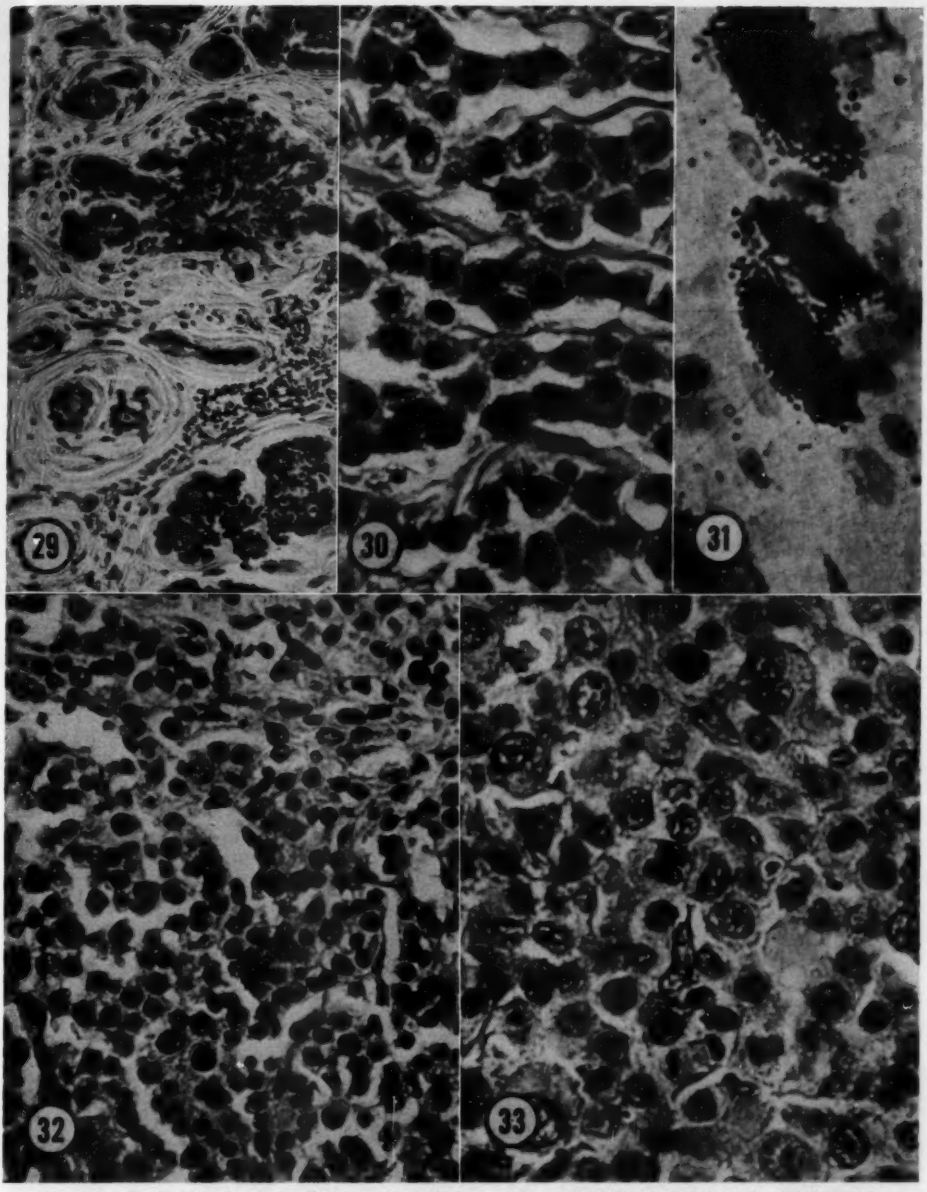

Fig. 29—Canine basal cell carcinoma. A small percentage exhibit hyalinization about the sweat gland-like epithelial structures.

Fig. 30—Canine mastocytoma. Tumor consists of large numbers of cells with round nuclei and abundant granular cytoplasm. A stain such as Giemsa's or toluidine blue must be employed to separate this from other round celled tumors.

Fig. 31—Canine mastocytoma. Stained with toluidine blue, the metachromatic granules in the cytoplasm may be easily seen.

Fig. 32—Canine transmissible venereal tumor. This tumor, usually transmitted by cell inoculation during coition consists of cells with round nuclei, abundant cytoplasm, some cellular pleomorphism.

Fig. 33—Canine transmissible venereal tumors. Note double and giant size nuclei as well as mitoses.

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It should be emphasized that, because many of the less well-differentiated mast cell lesions show few granules, standard procedure should be to stain all round cell sarcomatous neoplasms of the skin and oral mucous membrane with Giemsa's stain or toluidine blue to separate the mastocytomas. Malignant lymphoma is a rapidly fatal illness,[73] affected animals rarely living more than seven months. Microscopically, it may be lymphoblastic, lymphocytic, or reticulocytic. Our 6 examples of this tumor were in dogs, 5 of them more than 8 years of age. Three were lymphocytic, 2 lymphoblastic, and 1 reticulocytic.

Transmissible Venereal Tumor and "Myelocytoma."—The classification of round cell tumors of the canine skin and mucosa is in a state of confusion. Mastocytomas can usually be clearly segregated on the basis of metachromatic cytoplasmic granules in tumor cells and abundant associated eosinophils. However, a tumor that has been designated "myelocytoma" has been described as containing abundant eosinophils and somewhat similar tumor cells, except for lack of metachromatic cytoplasmic granules.[111]

There is a definite clinicopathological entity, the transmissible venereal tumor, that may occur extragenitally as well as genitally. A morphologically similar tumor occurs in the skin of dogs, usually as a raised "button," that has been designated variously as transmissible venereal tumor and histiocytoma. Thus, it is evident that many consider this skin lesion to be identical with the proved transplantable venereal lesion. Others question this, believing the two to be separate nosological entities. Further work is needed to clarify this matter which we are unable to resolve on the basis of our material. The transmissible venereal tumor is transmitted by direct transplantation of cells, usually in association with coition. The tumor commonly disappears spontaneously, leaving a state of relative immunity in the animals.[14, 56, 73, 102, 118]

Microscopically, differential diagnosis would include myelocytoma, lymphosarcoma, mastocytoma, aortic body tumor, and seminoma. Knowledge of its exact location will help to rule out some possibilities; all but the aortic body tumor and seminoma may be found in or about the mouth. Myelocytoma is a recently described, somewhat malignant, tumor that needs considerable further investigation[111] (fig. 32, 33; table 4).

Fibrosarcoma, Chondrosarcoma, and Osteosarcoma.—Fibrosarcoma was seen 18 times in our series; 11 in dogs, 4 in horses, 2 in mules, and 1 in a cow. Schlumberger,[104] in his review of oral neoplasms, cited 2 cases in horses but none in dogs. Cotchin[27] found 12 examples of "sarcoma" in 90 canine oral tumors, 9 of which occurred on the gingiva or palate. However, he did not specify the type of sarcoma. Mulligan[73] makes no mention of intraoral fibrosarcomas though his Plate XXXIII shows 1 from the submental region. Whether this was of dermal origin or a skin extension from a mandibular site was not disclosed. Riser[100] found these tumors in the mandibles of 3 dogs. Cotchin[24] found 17 of 84 canine neoplasms of the mouth, exclusive of the tonsillar area, to be sarcomas of undesignated type, all of the gingiva. Willis[125] observed a number of fibrosarcomas in animals, none of which occurred about the mouth. Judging from our cases and the available reports, the gingiva of the lower jaw and palate seem to be the more common sites.

Of special interest is a dermal fibrous tumor which Jackson and others[14, 52, 85]

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Fig. 34—Equine sarcoid. Resemblance to human dermatofibrosarcoma protuberans is marked; swirling of cell masses suggests neurogenic origin.

Fig. 35—Canine chondrosarcoma. Tumor is composed of immature cartilage-forming cells or chondroblasts and immature or poorly formed cartilage.

Fig. 36—Canine osteogenic sarcoma. Roentgenogram of lower canine jaw showing "sunburst effect" characteristic of osteogenic sarcoma. Note pathological fracture.

Fig. 37—Canine osteogenic sarcoma. This highly malignant jaw tumor is composed of numerous osteoblasts and spicules of immature neoplastic osteoid.

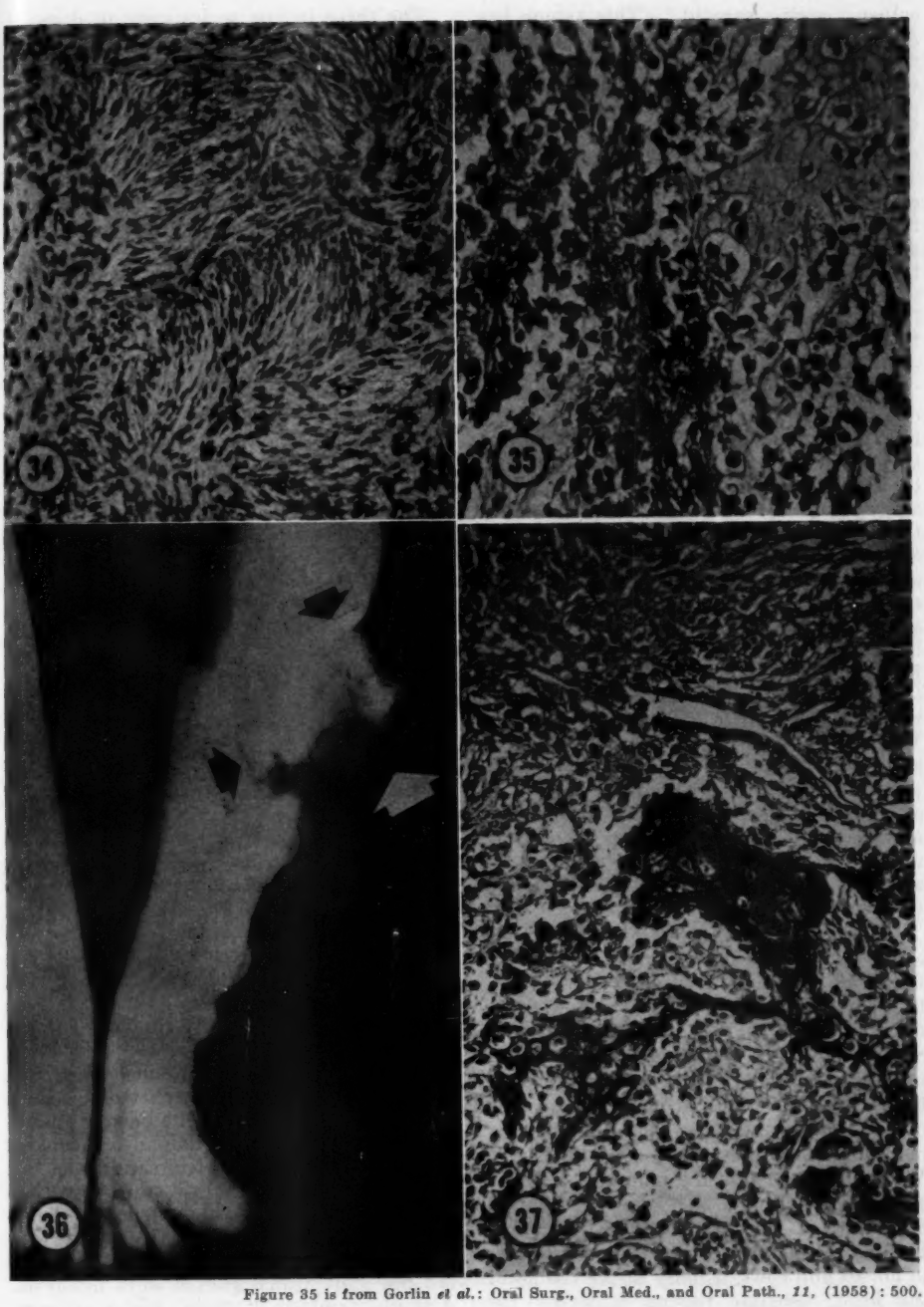

Figure 35 is from Gorlin *et al.*: Oral Surg., Oral Med., and Oral Path., *11*, (1958): 500.

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called "equine sarcoid." This tumor has a possible human counterpart in dermatofibrosarcoma protuberans. Both are of relatively low-grade malignancy and tend to recur locally. Metastasis is not observed. The overlying acanthotic epithelium covers a markedly cellular growth of fibroblasts. The whorling in some tumors strongly suggests neurofibromatous origin. Because of its clinical contagiousness, both natural and experimental,[85] it has been suggested that this lesion is caused by a virus. It has been reported that these lesions can be generated by inoculating a horse with a filtrate from bovine papillomas.[88] This tumor should be further investigated (fig. 34).

The 3 chondrosarcomas in our series all occurred on the palate and gingiva of dogs less than 8 years old. If the various general surveys reflect the true incidence of this tumor, it seldom occurs in animals. In some surveys it may have been included under the designation of "osteogenic sarcoma." Schlumberger[104] found none affecting oral structures. Mulligan[73] observed that these tumors are generally more common in the Shepherd breed, most commonly on the hard palate, nasal passages, and mandible. Feldman,[34] in reviewing the European literature, failed to find any cases involving the oral structures of domestic animals. Willis[125] described a case without metastasis that arose in the nasal cavity of a 6-year-old Cocker Spaniel. In our series, all of these tumors recurred after surgical removal. As in man, the tumor consists of immature, irregularly cellular cartilage in an anaplastic connective tissue stroma (fig. 35).

Osteosarcomas are apparently far more common than chondrosarcomas.[26] Cotchin[24] found 40 bone tumors in dogs to be of this type; 3 involved the mandible and 2 the maxilla. About half of the dogs were over 8 years old. The tumor metastasizes early in the clinical course of the disease, chiefly by way of the blood stream, much as in man.[28] Bone tumors in dogs are usually malignant and must be removed early.[100] Willis[125] found 1 in the palate of a Samoyede, and Neilsen *et al.*[80] reported 1 in the mandible of an 11-year-old Greyhound. Feldman[34] found reports of several cases in horses and cows, usually in the tissues of the head. Similar lesions involving the maxilla were reported[34] in the dog, sheep, and cat.

The three in our series were in dogs; 2 in the maxilla and 1 in the mandible (fig. 36, 37).

As in man, the tumor consists of immature, irregularly calcified spicules of new bone embedded in an anaplastic connective tissue stroma. Neoplastic multinucleated giant cells may be common. In very anaplastic tumors, the production of bone may be minimal.

Rhabdomyosarcoma.—Rhabdomyosarcoma is a rare tumor in animals and man. The authors have been unable to find any references to it in the oral region of domestic animals. It is highly malignant and metastasizes widely. Its cells are usually pleomorphic. They may be rounded or strap-shaped with several nuclei. Others are racquet-shaped with but one nucleus at the enlarged end. An occasional cell is markedly eosinophilic. Unless one finds cross striations or longitudinal myofibrils in a suspected rhabdomyoblast, one is extremely hard put to make this diagnosis. Many are so anaplastic that these features can not be demonstrated.

In our series, we felt but 1 tumor was compatible with the diagnosis of rhabdomyosarcoma. It arose in the cheek of a dog. In this case, striations could not be satisfactorily demonstrated but the cell types were strongly suggestive of this tumor. Two other tumors for a time placed in this category were reclassified as atypical histiocytomas.

Neoplasms and Cysts of the Major and Minor Salivary Glands.—Schlumberger,[104] in reviewing the literature and through personal communication, cited cases of adenoma, adenocarcinoma, mixed tumor, and duct papilloma in the dog. Mulligan[73] reported a "cancerous myoepithelioma" of the parotid gland and pointed out the marked similarity of these tumors to those of the canine mammary glands. Mixed tumors were reported by Plummer[94] in the cow and dog. Trotter[120] reported a case of "mixed-cell sarcoma" of the parotid gland in a survey of 305 malignant neoplasms in cattle. Sticker[113] reported carcinomas of the parotid gland in a cow and in 2 dogs. Riser[100] found 3 cases of "adenocarcinoma of salivary glands" in a series of 226 canine neoplasms of the mouth and nose. He did not state which glands were involved. A single neoplasm of canine major salivary glands was cited by Cotchin.[24,28] Its precise pathological nature was not specified.

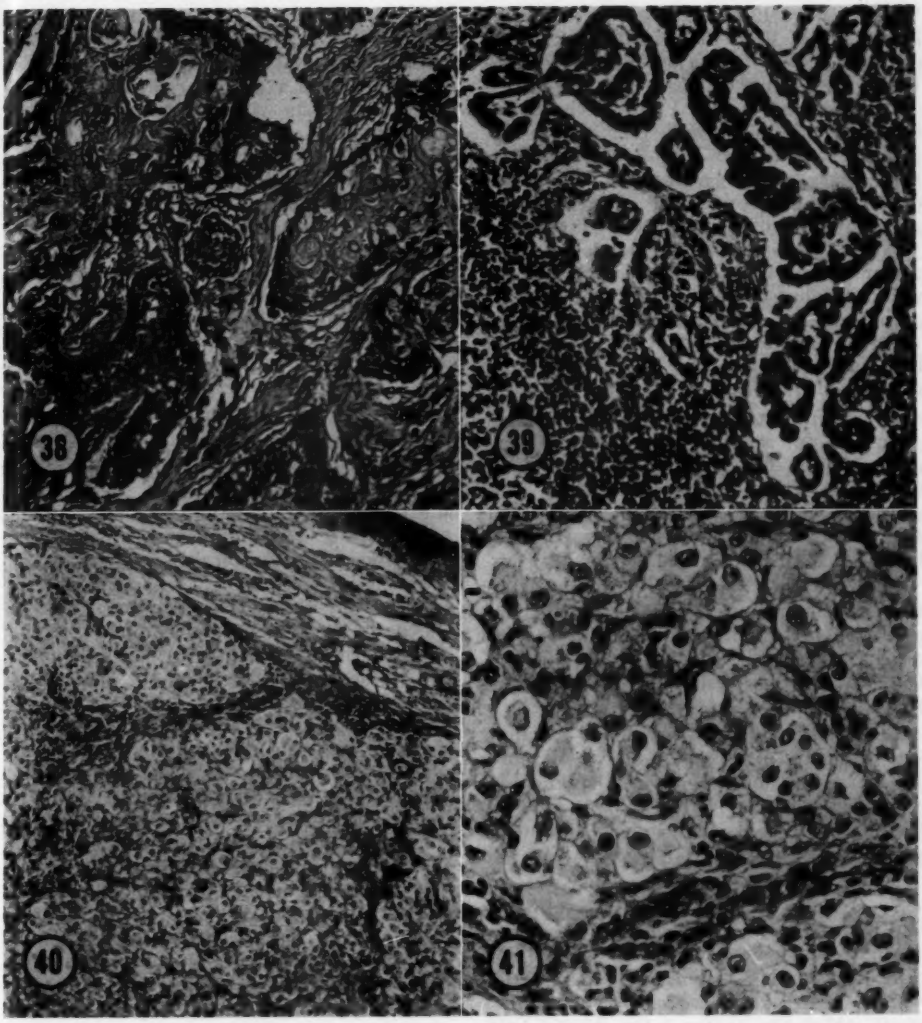

Fig. 38—Canine salivary gland carcinoma, squamous type. When the salivary glands are involved in a neoplastic process, chances of malignancy are great.

Fig. 39—Canine adenocarcinoma of minor salivary glands of palate metastatic to tonsil. The glandular pattern of the cancer cells can be easily seen.

Fig. 40—Feline alveolar soft part sarcoma. The tumor consists of large granular eosinophilic cells that are compartmentalized into a somewhat organoid structure.

Fig. 41—Feline alveolar soft part sarcoma. Higher power of slide seen in figure 40. Note abundant granular cytoplasm, small nucleus, well defined nucleolus.

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There were 12 salivary gland neoplasms in our series, 11 of them malignant. Eight of the 12 were in major salivary glands. Eight were found in dogs, and 1 each in a cat, horse, and sheep. Four of the malignancies could be classified as adenocarcinomas, 1 as acinic cell carcinoma, 1 as trabecular carcinoma, and the rest as undifferentiated carcinomas. There were an equal number of neoplasms arising in major and minor salivary glands. Of the major group, 5 of the 6 occurred in the parotid gland. The high rate of salivary gland malignancy seems to indicate that when a salivary gland tumor occurs, albeit rarely, chances of the tumor being malignant are great (fig. 38, 39).

In addition to the salivary gland neoplasms in our series, a pseudoparotid mass in a 10-year-old male Labrador—not included in this series—presented a diagnostic problem. It was reported by Gorlin and Clark [42] as a case of calcifying epithelioma of Malherbe.

Schlumberger,[104] citing Cole, reported an adenocarcinoma of a dog lip, and Mulligan [73] reported 1 in a canine tongue, both apparently of minor salivary gland origin. Cotchin [24,27,28] did not mention the occurrence of minor salivary gland tumors.

We observed 4 adenocarcinomas of minor salivary gland origin, 3 in dogs and 1 in a sheep. In addition, we observed single cases of mucous gland adenoma in a 10-year-old Shepherd Dog and hyperplasia of mucous glands in a 3-year-old Great Dane.

Cysts of salivary gland origin are apparently due to obstruction with foreign bodies, such as plant awns or kernels of grain which gain entrance and obstruct a duct. They may also cause inflammation which may result in a sialolith or salivary stone which can obstruct the ducts. Dilation of the obstructed duct, if it occurs in the floor of the mouth, produces a condition known as ranula.

Alveolar Soft Part Sarcoma.—One lesion we classified as alveolar soft part sarcoma, not without considerable reservation. This tumor apparently had not been reported previously in animals, although granular cell myoblastoma has been.[121] In man, it is exceedingly rare.[20,110] It has been called by several names, the most common ones being "malignant granular cell myoblastoma with organoid structure" and "alveolar soft part sarcoma."

Microscopically, the tumor cells are grouped or gathered together in discrete masses separated by delicate fibrous septa. The cells have a granular, at time vacuolated, cytoplasm with a distinct hyperchromatic nucleus.

Our case occurred initially on the upper gingiva of an 8-year-old female cat and recurred, extending to the posterior nasal cavity, over a period of five months. The microscopic appearance indicated an organoid granular cell tumor in part, but preservation of this specimen was poor and the diagnosis is, therefore, hesitatingly offered (fig. 40, 41).

DISCUSSION AND SUMMARY

The authors have reviewed 487 biopsied oral and pharyngeal lesions, both neoplastic and non-neoplastic, obtained from 377 dogs, 44 cattle, 35 cats, 17 horses, 9 mules, 3 sheep, 1 burro, and 1 pig. Among the non-neoplastic lesions were cases of pyogenic granuloma, actinomycosis, actinobacillosis, eosinophilic granuloma, trichinosis, globidiosis, and odontoma. The benign neoplastic lesions were fibromatous epulis of periodontal origin, canine oral papillomatosis, noncanine papillomata, and the oral papillomata of bovine hyperkeratosis, chondroma, lipoma, adenoma, and nevus.

The most common malignant oral neoplasm in this series was canine melanoma, whereas Withers and Cotchin in England found the squamous cell carcinoma most common. Other malignant neoplasms found in different animals were squamous cell carcinoma, basal cell carcinoma, carcinoma of the major and minor salivary glands, mast cell sarcoma, fibrosarcoma, chondrosarcoma, osteosarcoma, malignant lymphoma, rhabdomyosarcoma, transmissible venereal tumor, and "myelocytoma." The incidence, clinical features and the macro- and microscopic nature of these lesions were discussed in detail.

Lesions such as canine transmissible venereal tumor, "myelocytoma," and mast cell sarcoma require considerably further study. There is much to be learned concerning their life history, genesis, and inter-relationship, if any. It is a disadvantage to the histopathologist that once a poor prognosis is proffered by the laboratory, euthanasia is so frequently employed, limiting follow-up.[124]

This study has raised many questions. Certainly a virgin field, animal oral path-

ology offers countless opportunities for research. Listed below are but a few of the myriad questions that can not be answered:

1) What is the exact mechanism of oral ulceration in renal dysfunction?

2) What role, if any, do bacteria really play in pyogenic granulomas? Is loss of local resistance a factor?

3) What is the nature of eosinophilic granulomas in animals? Is it a cholesterol histiocytosis as in man or is it a reaction to an insect bite or parasite or something entirely different? What is its relationship to eosinophilic myositis if any exists? Are the diseases described as eosinophilic myositis in dogs and cattle the same?

4) What is the true incidence of spontaneous neoplasia in animals that are slaughtered before they reach "cancer age?" What effect if any does geography have upon oral cancer in domesticated animals?

5) Is oral cancer really much more common in dogs, or do other factors becloud the statistics?

6) What etiological factors are responsible for the fibromatous epulis of periodontal origin? How important is breed? How common is it in other species? Is erroneous diagnosis of variants of this lesion responsible for nearly all reports of ameloblastoma in dogs?

7) What counterparts of human "neoplasms of odontogenic origin" occur in animals?

8) Does equine or bovine dental caries have a human counterpart? Is the cervical caries seen in sheep related to tooth form?

9) Why is periodontal disease so common in the horse? Why is it so uncommon in the cat?

10) In view of our observations, should we not discard the concept of melanoma not rising from junctional lesions? Is the so-called "benign melanoma" not a melanoma at all?

11) Why doesn't leukoplakia occur as a premalignant lesion in animals as it does in man?

12) What relationship does "myelocytoma" have to mast cell sarcoma and other round cell neoplasms? What is its life history?

13) Why are nearly all animal salivary gland tumors malignant?

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XUM

The Absorption of Red Blood Cells After Parenteral Injection at Various Sites

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THE ABSORPTION of particulate matter and red blood cells from the peritoneum has been studied for many years. Cunningham,[6] by transfusing avian red blood cells into cats, was probably the first to observe that red blood cells were absorbed from the peritoneal cavity. Histological studies revealed the presence of avian cells in the circulating blood of these cats.

At a later date, Siperstein and Sansby[12] obtained evidence of red blood cell absorption from the peritoneal cavity of the rabbit. This discovery prompted them to use intraperitoneal transfusions clinically in children. Using radioiron as a red blood cell tagging agent, Hahn[7] demonstrated that intraperitoneal transfusions in normal dogs resulted in absorption of 20 to 100 per cent of the transfused cells within the first twenty four hours. Transfusion of anemic dogs by this method resulted in absorption of 5 to 80 per cent of the transfused cells in the same period of observation. McKee and Stewart,[9] using radioiron as a tagging agent for intraperitoneally transfused red blood cells, obtained data indicating an absorption of these tagged cells of from 26 to 67 per cent in 3 ascitic dogs.

Courtice *et al.*[4] demonstrated that red blood cells absorbed from the peritoneal cavity are returned to the systemic circulation via the right lymphatic duct, rather than the thoracic duct. Crosby[5] found that the absorption of intact red blood cells following peritoneal, pleural, subcutaneous, and intramuscular transfusions in rabbits ranged from 55 to 90 per cent, 20 to 35 per cent, 5 per cent, and 5 per cent, respectively. Pritchard and Weisman[11] and Pritchard and Adams[10] found that intact red blood cells were absorbed from the human peritoneal cavity.

There is little doubt that red blood cells are absorbed from the peritoneal cavity. There remains a question as to the rate of red blood cell absorption, the actual quantity absorbed, and the effect of diseases upon these factors. This investigation was carried out in order to obtain further information on these points.

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MATERIALS AND METHODS

The dogs used in these experiments were mongrels, with the exception of 7 Beagle pups from two litters of 3 and 4 each.

All dogs were fed a commercial ration that was considered adequate for mature dogs. This same ration, with 10 per cent powdered milk added, was given to the pups. Adult dogs were fed daily, whereas pups were given multiple feedings according to their need. Water was given *ad libitum*. This method of feeding maintained all experimental animals in a good state of nutrition.

A common kennel room, containing individual cages of similar design in which the dogs were housed at random, was used throughout the period of experimentation.

All animals, unless otherwise stated, were free from internal parasites as determined by biweekly fecal examinations performed throughout the experimental period. Before dogs were used in the experiment, they had to have four successive negative fecal flotations. However, one group of dogs with varying degrees of hookworm infection and other diseases was studied for comparative results (table 2).

In order to determine whether the red blood cells injected passed into the peripheral circulation, they were tagged so that they could be distinguished from the red blood cells already in the circulation. In this work, the cells were tagged with sodium radiochromate (Cr^{51}) by a modification of the Sterling and Gray method.[13]

The number of cells absorbed was calculated by knowing the volume and total radioactivity of the tagged cells injected, the blood volume of the dog, and the radioactivity per milliliter of blood. The volume of tagged cells in the circulation may be calculated from these factors, and the amount of red cells picked up from any area may be determined.

Method for Tagging and Counting Red Blood Cells.—The blood to be transfused was mixed with $Na_2Cr^{51}O_4$ and incubated at 39 C. for 45 to 60 minutes. During incubation the blood was swirled gently, approximately every 10 minutes. This procedure resulted in an absorption by the red blood cells of 90 ± 5 per cent of the radiochromate. As soon as tagging was complete, 1 mg. of ascorbic acid was added for each 1 ml. of blood, after which it was incubated for an additional 15 minutes. The ascorbic acid converted the chromate ion, which readily enters the red blood cells, to the chromic ion, which enters red blood cells to a very limited degree. This was done to prevent the radiochromium remaining in the plasma from tagging

the red blood cells *in vivo*. Several experiments were performed in order to determine the value of the ascorbic acid step, and it was found that it could be omitted without increasing the counting error.

After tagging, the blood was injected by the desired route. The volume of radioactive red blood cells that entered the circulation was measured by counting either 1 or 2 ml. of blood. One milliliter samples were counted in small pups, and 2 ml. samples were counted in larger pups and mature dogs. It is important that the same volume of red blood cells be counted each time; otherwise hemodilution or hemoconcentration may lead to erroneous conclusions. The procedure used was to count 1 or 2 ml. of blood and then calculate the volume of red blood cells by obtaining a hematocrit reading. At the end of the experiments, all counts on the same dog were corrected to a mean hematocrit value. Wintrobe hematocrit tubes were centrifuged at 4,000 r.p.m. at a radius of 9 cm. for 30 minutes. The error in hematocrit readings, due to plasma trapped in the column of packed red blood cells, was determined to be 1 per cent by using radioiodine (I^{131}) tagged serum albumin.[1, 6] All hematocrit readings were corrected accordingly.

At the beginning of each experiment, it was necessary to determine the radioactivity of the plasma fraction of the chromium-tagged blood used for transfusion so that the radioactivity of the red blood cells could be calculated. This was accomplished by counting aliquots of plasma. The hematocrit reading was then used to calculate the total plasma count of the blood as well as the cell activity. A similar correction was applied to blood samples from the recipient for the first 72 hours. After this time, the plasma counts were negligible.

A deep-well scintillation detector connected to a single-channel spectrometer* was used for all counting. When large volumes of blood are counted in a crystal of this type, it is possible that settling of the blood cells may alter the count. The reason for this altered count is that in thoroughly mixed blood samples there are many radioactive red blood cells above or near the top of the crystal but, as settling occurs (either while counting or in a sample that has remained in a rack for a period of time), the radioactivity moves nearer the center of the crystal. This would improve the geometry of the crystal and thereby yield higher counts. In order to determine the importance of settling in our crystal and of the sample sizes used throughout the experiments, a study of 42 blood samples was performed. The samples were counted after thorough mixing and then they were centrifuged and recounted. All duplicate counts were within 1 per cent, which was considered to be the reproducibility of the instrument. It was concluded that settling was an unimportant factor, considering the amounts of blood and the size of crystal used in these experiments.

* Nuclear Chicago Spectrometer, Model 132 with a deep-well thallium-activated sodium iodide crystal, manufactured by Nuclear Chicago Corp., Chicago.

Methods for Determining Blood Volume.—Blood volumes were determined by the use of T 1824 (Evans blue) and radiochromium-tagged red blood cell dilution.[5]

EXPERIMENTAL PROCEDURE AND RESULTS

Red blood cells tagged with radiochromium were injected subcutaneously, intramuscularly, intraperitoneally, and into the medullary cavity of the femur. The subcutaneous injections were given in the flank area; the intramuscular injections in the rectus and oblique muscles of the abdominal wall and in the quadriceps muscle; and the intramedullary injections in the medullary cavity of the femur. The intraperitoneal injections were made on the right side anterior to the umbilicus, one to two inches lateral to the midline. This area was selected to reduce the possibilities of injection into an abdominal viscus. Even so, four intraperitoneal transfusions failed. It was found that injections had been made into the abdominal wall (2 dogs), the stomach (1 dog), and the small intestine (1 dog).

Types of Blood and Cell Suspensions Used.—With the exception of four transfusions of tagged red blood cells suspended in normal saline solution, all transfusions were made with whole blood tagged with radiochromium. Isotransfusion† and autotransfusion methods were used. The results of these experiments are summarized (table 1). With the exception of the subcutaneous and the intramuscular routes of administration, significant numbers of the transfused cells entered the circulation.

Effects of Disease on Absorption of Red Blood Cells from the Peritoneum.—Intraperitoneal transfusions of chromium-tagged blood were made in dogs with subclinical hookworm infection, clinical hookworm infection, and canine distemper in order to evaluate the effects of these conditions on the absorption of red blood cells from this site. A dog was considered to have clinical hookworm infection if a fecal flotation revealed the presence of eggs of this parasite and the hematocrit reading was below 25 per cent. A subclinical classification was made if a fecal flotation revealed the presence of these eggs, but the hematocrit value

† The word isotransfusion is used to identify a type of transfusion in which blood was withdrawn from an individual, tagged with radiochromium, and transfused into another individual of the same species, while autotransfusion is used to identify a transfusion in which an animal is transfused with his own blood.

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TABLE 1—The Uptake of Red Blood Cells After Various Transfusion Methods

Method of transfusion	Mean uptake (%)	Standard error of mean	Standard deviation	No. of dogs
INTRAPERITONEAL				
Autotransfusion				
Adults*	86.1	3.24	13.3	17
Adults†	83.4	2.9	12.6	19
Pups†	80.3	4.3	18.4	22
Isotransfusion				
Adults*	78.6	3.84	18.8	24
INTRAMEDULLARY				
Autotransfusion			Range in per cent	
Adults	95.3	.....	93–97.9	3
SUBCUTANEOUS				
Autotransfusion				
Adults	2.0	.....	1.0–3.0	4
INTRAMUSCULAR				
Autotransfusion				
Adults	3.8	.....	2.0–6.0	4
INTRAPERITONEAL				
Saline-solution suspended cells				
Adults	77.0	.....	51.0–99.0	4

* Mongrels in average condition and not necessary parasite-free. † Mongrels in good condition and free from detectable disease and parasites.

was 25 per cent or above. The diagnosis of distemper in 3 dogs was made from clinical findings only. The results of this study were tabulated (table 2). Apparently, hookworm parasitism and distemper did not materially affect the uptake of red blood cells from the peritoneal cavity.

TABLE 2—The Maximum Uptake of Intraperitoneally Transfused Red Cells in Dogs with Various Diseases

Disease	Maximum mean uptake (%)	Range (%)	No. of dogs
Distemper	88.3	88.3–97.0	4
Subclinical hookworm infection	90.1	78.0–100.0	6
Clinical hookworm infection	78.0	54.0–91.0	6

Rate of Uptake of Red Blood Cells.—In order to determine how rapidly red blood cells were absorbed from the peritoneal and intramedullary cavities, blood samples were drawn from dogs and counted at frequent intervals during the first and second post-transfusion days and again at one week following transfusion.

The results of the intraperitoneal transfusions are given (table 3). These data indicate that the rate of uptake was quicker during the first 24 hours than any subsequent 24-hour period. The maximum amount of uptake of the red blood cells was reached in ten to 14 days.

In 3 average-sized dogs, intramedullary transfusions of approximately 50 cc. each of chromium-tagged blood resulted in a slight delay in the appearance of this blood into the systemic circulation. The maximum uptakes of 95.0, 97.9, and 93.0 per cent were obtained within five minutes post-transfusion. The time required for transfusion was 45, 60, and 65 minutes, respectively.

One experiment was designed to compare the relative values of intraperitoneal and intravenous transfusions of blood. This was accomplished by using 4 Beagle siblings that were 10 weeks old. The weight range of these pups was from 8.5 to 10.0 lb.; the mean weight was 9.3 lb. They were in good health and free from internal parasites. Approximately 20 cc. of blood was withdrawn from each and tagged with radiochromium. Three milliliters of this blood was set aside for hematological study. Two pups were selected at random to receive intravenous transfusions. Autotransfusions of approximately 17 cc. of blood were given each pup. From the re-

TABLE 3—The Rate of Absorption of Red Blood Cells from the Peritoneal Cavity

Time from end of transfusion	Av. absorption of red blood cells (% of total)	Range (%)	No. of dogs
30 min.	0.6	0.3–0.8	6
2½ hr.	4.7	1.7–9.0	4
4 hr.	9.3	5.0–18.0	3
7½ hr.	25.0	5.0–45.0	7
17 hr.	33.0	15.0–47.0	5
24 hr.	47.0	14.0–64.0	41
2 days	63.0	40.0–73.0	41
3 days	76.0	62.0–89.0	41
1 week	80.0	70.0–100	41
10–20 days	81.7	58.0–100	41

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sults (graph 1), it can be seen that the radioactivity of the peripheral blood of the pups receiving the intraperitoneal transfusions approaches, within ten days to two weeks, that of the intravenous transfusions.

Subcutaneous and Intramuscular Absorption.—Red blood cells transfused into the subcutaneous tissues and the muscles are poorly absorbed. In 4 dogs studied, the amount of absorption of red blood cells from these locations ranged from 1 to 6 per cent, with a mean absorption of less than 3 per cent. The dissipation of residual radioactivity at the site of injection was measured biweekly. By studying simultaneously the disappearance of the radioactivity over the site of injection and the radioactivity of the blood, it was determined that less than 3 per cent of the injected red blood cells were absorbed intact. Over 97 per cent of the red blood cells were destroyed at the site of injection.

DISCUSSION

These studies indicate that in the average dog, young or mature, the mean absorption of red blood cells from the peritoneal cavity is 81.7 per cent. The cells are apparently absorbed as intact, functional units. It is difficult to explain the loss of the remaining 18.3 per cent of cells.

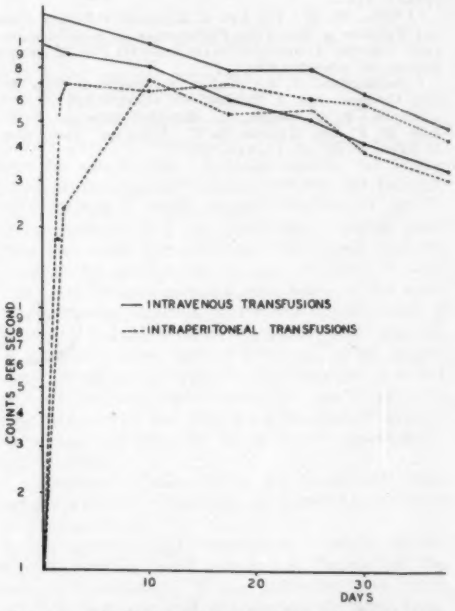
The work of Courtice [4] offers a plausible explanation for this loss. He reported that red blood cells, after absorption from the peritoneal cavity, returned to the circulation through the right lymphatic duct rather than the thoracic duct. The macroscopic anatomy of this region suggests that they must pass through an extensive system of lymph nodes. He found the sinuses of these lymph nodes to be completely filled with large numbers of macrophages that were engorged with red cells, and there was evidence of subpleural extravasation along the parasternal lymphatics.

Other factors that may contribute to the destruction of red blood cells are mechanical injury during handling, the deleterious effects of anticoagulants, the development of antibodies, or hemolysis by bacteria.[10]

The clinical use of intraperitoneal transfusions of whole blood for severe hookworm anemia would perhaps be of value since these dogs were depleted of plasma proteins as well as red blood cells. Absorption from the peritoneal cavity begins soon after transfusion and continues at a fairly rapid rate for approximately two days, thus simulating a continuous intravenous transfusion.

SUMMARY

1) In 41 dogs, 81.7 per cent of the red blood cells given intraperitoneally were absorbed intact.



Graph 1—Radiochromium-tagged red blood cells were given intraperitoneally to 2 of 4 Beagle litter mates and intravenously to the other 2. The rapid uptake of red blood cells from the peritoneal cavity, reaching a maximum in approximately 15 days, is evident.

2) An average of less than 3 per cent of the red blood cells injected intramuscularly and subcutaneously were absorbed.

3) An average of 95.3 per cent of the red blood cells transfused into the medullary cavity entered the general circulation intact.

4) The average rate of intraperitoneal absorption of red blood cells is 48.0 per cent in 24 hours, 65.0 per cent in 48 hours, and up to an average of 81.7 per cent in one to two weeks.

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A Comparison of Blood Volumes as Measured by Rose Bengal, T-1824 (Evans Blue), Radiochromium-Tagged Erythrocytes, and a Combination of the Latter Two

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THE DEGREE of filling of the spleen of dogs will vary the venous hematocrit reading without materially altering the blood volume, according to Reeve.[4] This indicates that any method for measuring blood volume that depends on the venous hematocrit determination is likely to be in error. Therefore, blood volumes obtained in the present experiment by using rose bengal, T-1824, and radiochromium-tagged cells were compared to those obtained by the combination of T-1824 and radiochromium-tagged cells. All of these methods were then related to the body weight. The purpose of this study was to determine normal blood volumes in healthy dogs and to determine the accuracy of several methods of blood volume measurement.

MATERIALS AND METHODS

Rose Bengal Method.—The rose bengal method employed was a modification of the original method of Simpson.[6] A blood sample was drawn, prior to the injection of the rose bengal, to be used in preparing the blank plasma sample and the standard rose bengal solution. One milliliter of 5 per cent rose bengal was injected intravenously into each dog, regardless of size. Blood samples were drawn at intervals of four, eight, and 12 minutes. The plasma was compared with the rose bengal standard at 547 mμ. The spectrophotometer* was set at 100 per cent transmittance with the blank serum sample in order to correct for any coloring matter in the plasma. The standard was prepared by dissolving rose bengal in the plasma. Thus, any effects that the plasma might have on the spectral absorption of rose bengal were compensated.

The optical densities of the plasma samples were plotted on semilogarithmic paper and the density at zero time was obtained by extrapolation. With this figure, the plasma volume was obtained by the following formula, where O.D.S. is the optical density of the standard and O.D.P. is the optical density of the plasma samples extrapolated to zero time:

$$\frac{\text{O.D.S.}}{\text{O.D.P.}} \times \text{dilution of standard} = \text{plasma volume}$$

The blood volume was then calculated from the venous hematocrit reading.

T-1824 Method.—The general method for determining plasma volume by T-1824 was similar to that for rose bengal. A blood sample was drawn prior to the injection of the dye for the purpose of preparing a blank plasma sample and the T-1824 standard. A 1 per cent T-1824 aqueous solution was used for injection, and blood samples were drawn at intervals of five, ten, and 15 minutes. The plasma samples were read on the spectrophotometer against a plasma blank and a standard T-1824 solution prepared with plasma. The samples were read at 627.5 mμ.[1] The optical densities of the plasma samples were extrapolated to zero time and the formula for rose bengal was used to calculate the plasma volume. Blood volumes were then calculated by using the venous hematocrit readings.

Chromium^{51} Method.—The red blood cells were tagged with radiochromium by a method described in another paper.[3]

Ten minutes after intravenous injection of the tagged blood, a blood sample was drawn and the cells were counted. It was unnecessary to draw multiple samples and extrapolate to zero time. Samples drawn at intervals of five, ten, and 30 minutes yielded the same counts within the experimental error of the procedure. This was undoubtedly due to the minute loss of red blood cells from circulation during the first few minutes after injection. All samples were corrected for plasma counts. This was done by counting a sample of plasma and then calculating the total plasma count from the hematocrit reading.

The total volume of red blood cells was then calculated by the following formula:

$$\frac{\text{total red blood cell counts}}{\text{red cell counts per ml.}} = \text{total volume of the red blood cells}$$

Total red blood cell counts = the total counts of the blood injected, corrected for plasma counts.

Red blood cell counts per milliliter = the count obtained from 1 ml. of red blood cells. This figure was obtained by counting 2 ml. of whole blood and then obtaining a hematocrit value on the same blood. From this figure the volume of red blood cells counted was calculated. The counts per milliliter of red blood cells were then obtained by this formula:

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* Coleman Universal Spectrophotometer, manufactured by Coleman Instruments, Inc., Maywood, Ill.

$$\frac{\text{total volume of red blood cells counted}}{1 \text{ cc.}} :: \frac{\text{counts obtained}}{X}$$

X = counts per ml. of red blood cells. The packed red blood cell volume as obtained by the hematocrit value must be multiplied by the number of milliliters of whole blood counted in order to obtain total volume of red blood cells.

Total Blood Volume Determination.—Blood volumes were determined by combining the plasma volume obtained by T-1824 and the cell volume obtained by radiochromium-tagged red blood cells. For these measurements, radiochromium-tagged red blood cells and T-1824 were injected simultaneously. Blood samples drawn at five, ten, and 20 minutes postinjection were used to determine the dilutions of T-1824 and chromium-tagged red blood cells. Procedures outlined previously were used to determine plasma and red blood cell volumes. The results obtained for these separate components were then added to obtain the total blood volume. The weight and blood volume of each dog were determined and the blood volume was then converted to a percentage of body weight.

RESULTS

Blood volumes obtained by combining the red blood cell volume, as measured by radiochromium, and the plasma volume, as measured with T-1824, were smaller than those obtained by dye dilution and hematocrit methods. Blood volumes obtained by using the radiochromium-tagged cells and the venous hematocrit values were less than volumes obtained from the combined red blood cell dilution and T-1824 methods.

The data from these various methods of blood volume determination (table 1) indicate that the dye dilution techniques, when used alone, render larger blood volumes than are obtained from Cr^{51}-tagged cell dilution or the combined method.

TABLE 1—Blood Volumes as Determined by Various Methods

Method	Percentage of body weight	Standard deviation	S. E. mean*	No. of dogs
T-1824	8.72	1.8	0.29	39
Rose bengal	9.19	1.23	0.41	9
Cr^{51}-tagged cells	7.07	0.97	0.24	17
T-1824, Cr^{51} combination	8.1	0.43	0.046	41

* S.E. = standard error of the mean.

DISCUSSION

The ratio of F-cells has been defined by Reeve [4] as the over-all cell percentage divided by the venous cell percentage. If the ratio of cells to plasma was perfectly homogeneous throughout the body, then the F-cell ratio would be equal to one. In this case, blood volumes obtained by all of the methods described in this paper should be the same. Since the blood volumes are not the same, the F-cell ratio is not equal to one. The mean F-cell ratio, as calculated from all of our experiments, is 0.895. This figure is close to that obtained by Reeve [4] from splenectomized dogs. From this it would seem that the spleens of the dogs were greatly constricted when blood volume measurements were made.

Blood volumes obtained by the rose bengal method were, on the average, 6 per cent higher than those obtained by T-1824. Sear [5] made simultaneous measurements with T-1824 and radioiodine-tagged serum albumin and found that they gave results within 0.5 per cent. Allen [2] made simultaneous measurements with hemoglobin and T-1824 and found that these agreed closely. From this evidence, it seems likely that T-1824 gives an accurate plasma volume and that rose bengal plasma volume is high.

SUMMARY

A comparison of blood volumes measured by T-1824, rose bengal, red blood cells tagged with radiochromium, and a combination of T-1824 and radiochromium-tagged cells yielded blood volumes of 8.72, 9.19, 7.07, and 8.1 per cent of the body weight, respectively.

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The Effects of Certain Diseases and Transfusion Methods on the Life Span of Red Blood Cells

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THE PURPOSE of this investigation was to determine what effects certain diseases, methods of blood transfusion, and age had on the viability of red blood cells. Various investigators have studied the life span of red blood cells by tagging them with Fe^{59}, N^{15}, C^{14}-lysine, Cr^{51}, and by the differential agglutination technique.[1, 2, 4-8] The results obtained by these methods indicate that the life span of canine red blood cells varies from 90 to 120 days. These are normal values for normal healthy dogs. The effects of diseases, blood transfusion methods, and age on the life span are largely unknown.

MATERIALS AND METHODS

For this study, the red blood cells were tagged with 59 to 100 μc. of sodium radiochromate by a method previously described.[3] After transfusion of the tagged cells, counts were made on identical quantities of red blood cells at biweekly intervals. A standard prepared with sodium radiochromate (Cr^{51}) was counted at each observation period. This standard was used to correct the counts obtained from blood samples for the physical decay and variations in the counting instrument. These blood counts, after correction, were converted to a percentage of the original count. These percentages were then plotted on semilogarithmic or linear graph paper. We believe that semilogarithmic plots are easier to read; however, the difference is not great and either will give the same results. The life span of a particular group of red blood cells was taken to be that point when the radioactivity of the blood sample completely disappeared.

A deep well scintillation detector coupled to a single channel spectrometer was used for all of the counting.*

EXPERIMENTAL PROCEDURE

The life span of red blood cells was studied in 78 different dogs. All dogs used to determine the life span of red blood cells were free of parasites and in healthy condition. Fecal flotations were performed biweekly throughout the experimental period and any animal found to have parasitic ova was removed from the experiment. The life span of red blood cells in one group of dogs infected with hookworms was studied for comparative purposes.

One group of dogs was given intravenous autotransfusions in order to establish the normal life span of red blood cells as measured by these procedures and equipment. Autotransfusions rather then isotransfusions were used to avoid the possibility of blood incompatibilities and a shortened red blood cell life span. Other dogs were given intraperitoneal auto- and isotransfusions and intramedullary autotransfusions. The intramedulllary transfusions were injeced into the medullary cavity of the femur. The red blood cell life span was also studied in dogs with clinical and subclinical infections of hookworms.

The group of animals that was subclinically infected with hookworms consisted of 12 dogs in good health and with no signs of enteritis. Their hematocrit readings were consistently 25 per cent or above.

A second group of 8 dogs was considered to have clinical hookworm infections. They were thin, anemic, and had a considerable degree of lassitude. Hemorrhagic diarrhea was present but was not a constant finding. Fecal flotations on these dogs revealed large numbers of hookworm eggs. The hematocrit readings of this group varied from 8 to 23 per cent.

The effects of canine distemper on red blood cell life was studied in 2 dogs, 1 adult and 1 pup. These cases were diagnosed clinically and were not confirmed by serological or histopathological studies.

RESULTS

A statistical analysis of the means of the life spans of the red blood cells given intravenously and intraperitoneally indicated that there was no significant difference. This indicates that the red blood cells absorbed from the peritoneal cavity are not harmed enough to shorten their life span.

Neither was there any significant difference between the iso- and autointraperitoneal transfusions. The blood used for the isotransfusions was known to be compatible. Any incompatibility in the transfused blood would have greatly reduced the life span.

The red blood cells apparently had a slightly shorter life span in pups as com-

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* Nuclear Chicago Spectrometer, Model 132, with a deep well thallium activated NaI crystal.

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pared with adults. This difference proved to be statistically significant.

Both clinical and subclinical hookworm infections significantly reduced the life span of the red blood cells.

DISCUSSION

Measuring the life span of red blood cells by using radiochromium has been studied by Stohlman,[5] Ebaugh,[4] and others. According to these authors, the chromium activity leaves the blood by physical decay of chromium^{51}, elution of the chromium from the red blood cells, and normal attrition of red blood cells. Physical decay of the chromium can be corrected with little difficulty. This leaves two other factors which cause a loss of chromium from the blood; *i.e.*, elution and red blood cell destruction. According to Stohlman, elution is a semilogarithmic function and red blood cell destruction is a linear function. This complication makes it difficult to determine, with any degree of accuracy, the rate of red blood cell destruction by making several observations and then to determine the life span by extrapolation. The plots obtained are neither semilogarithmic nor linear. The life span can be obtained with a degree of accuracy by counting the activity of the blood until it completely disappears. The end point of the disappearance of radioactivity from the red blood cells can be taken as the end of their life span. All life span measurements in this study were determined in this manner. Life spans of red blood cells measured by this technique compare favorably with those determined by other methods.

One of the difficulties encountered in measuring the red blood cell life span in pups was that as they grew, their blood volume increased. This constantly increasing blood volume would cause a dilution of the chromium-tagged cells and the red blood cells would seem to be disappearing from the blood at a faster rate than they actually were. It is conceivable that this dilution may have reduced the blood counts enough to give a slight artificial shortening of the life span. From this, it seems possible that the life span of red blood cells in pups may not actually be shorter than it is in adults.

CONCLUSIONS

The life spans of red blood cells that are transfused by intraperitoneal, intravenous, and intramedullary routes are essentially the same. Hookworm infections, even though subclinical in nature, cause a definite reduction in the life span of the red blood cells. This is undoubtedly due to the increased loss of red blood cells through the parasites.

TABLE 1—The Effect of Various Factors on the Life Span of Red Blood Cells

Method of transfusion or disease	Mean life span (days)	Standard deviation	Standard error of mean	No. of dogs
Parasite-free dogs				
Intravenous autotransfusion	104.1	10.4	3.17	11
Intraperitoneal autotransfusion	98.0	8.92	3.36	7
Intraperitoneal isotransfusion	96.5	11.6	4.41	7
Auto- and isointraperitoneal transfusions combined	97.5	9.79	2.64	14
Adult dogs	105.2	15.8	4.23	14
Pups	98.8	10.5	2.82	14
Intramedullary transfusions	108.6	range 97.6–113.0 days	.....	3
Dogs with canine distemper infection	87.0	range 71.0– 93.0 days	.....	2
Dogs with subclinical hookworm parasitism	92.9	23.3	6.71	12
Dogs with clinical hookworm parasitism	21.7	9.8	3.46	8

SUMMARY

1) The mean life span of red blood cells in adult dogs and pups in 105.2 and 98.8 days, respectively.

2) The life spans of red blood cells transfused by intraperitoneal, intravenous, and intramedullary routes are 97.5, 104.1, and 108.6 days, respectively.

3) The life span of red blood cells in dogs with clinical and subclinical hookworm infections is 21.7 and 92.9 days, respectively.

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Nephelometry and a Nephelo-Culture Flask Used in Measuring Growth of Leptospires

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THE GROWTH PATTERN of Leptospira has been sudied by Chang,[1,2] using direct microscopic counts, and Greene *et al.*,[8] using a Klett-Summerson photoelectric colorimeter for measurement of total growth response to various amino acids, vitamins, and proteins. Dry-weight measurements have been used to characterize total cell crops of Leptospira[12] and to quantitate optimal antigen densities.[9] Fulton and Spooner[6] characterized the assimilative process of *Leptospira icterohaemorrhagiae* by relating the yield in percentage of bacterial nitrogen to the nitrogen per milliliter of medium. Of the methods to date, dry weights and microscopic counts have been used most frequently.

According to Monod,[10] dry weights are the most direct to carry out, although they are cumbersome. He reported that the dry-weight technique is most accurate when large amounts of cells are used and recommended that this method be used as a check upon indirect methods of estimating growth, such as turbidimetry. Although few investigators have reported the use of turbidimetric methods to study the growth response of Leptospira, it was felt that an optical measurement would be the method of choice. With Leptospira, the small numbers and the more gradual changes in growth lend themselves to measurement by nephelometry rather than by light transmission methods. The use of nephelometry as an aid in studying the growth of slow-growing bacteria was suggested by the work of Fisher and Kirchheimer[4] and of Fisher *et al.*[5] These investigators found that turbidimetry by nephelometry was a method of high precision and sensitivity and that instrument response was directly related to bacterial numbers.

The light absorbency of Stuart's medium containing serum is influenced directly by the degree of hemolysis of the serum enrichment. Distinctive absorption occurs at 416, 540, and 580 mμ in the visible region of the spectrum. Optical density values at 416 mμ can vary from 0.01 to 1.0, or even higher, and range from 0.02 to 0.45 at both 540 and 580 mμ. Since nephelometry does not measure absorbed or transmitted light, one is not concerned with the highly variable aspect of color. This fact further suggests the use of scattered light to measure Leptospira growth responses.

Most studies of the growth pattern of *Leptospira pomona* have been carried out in conventional 20- by 150-mm. test tubes and 250-ml. Erlenmeyer flasks. The growth was measured nephelometrically in optical cuvettes at regular intervals. Cohen and Barner[3] modified an aeration culture cylinder to make direct photoelectric colorimeter readings in their growth studies of a thymine-requiring strain of *Escherichia coli*. Recently, however, a nephelo-culture flask has become available. Although this flask has been described in commercial literature and was shown publicly in 1956, no references to it and its potential application to microbiology have appeared in the literature. The unit is a combined culture flask and sidearm optical cuvette which has the possibility of offering a simple and practical method of measuring bacterial turbidity.

It is the purpose of this report to point out some of the practical advantages of the nephelo-culture flask and the close agreement with data obtained when similar growth assays were carried out in conventional test tubes and Erlenmeyer culture units. It is hoped that the description of the nephelometric procedures employed will further emphasize the unique value of this method in turbidimetrically estimating the growth of Leptospira.

MATERIALS AND METHODS

Leptospira pomona (Johnson) was used in this study and has been used regularly since January, 1955, as a nutritional assay organism and as the antigen strain for various serologic studies. Com-

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TABLE 1—Identity and Source of Leptospira Cultures

Species	Strain	Source
L. pomona	Johnson	Dr. H. G. Stoenner, U.S.P.H.S., Rocky Mt. Lab., Hamilton, Mont.
L. pomona	suis	Div. Vet. Med., Walter Reed Army Med. Cen., Washington, D.C.
L. pomona	Pomona	Div. Vet. Med., Walter Reed Army Med. Cen., Washington, D.C.
L. pomona	lamb	Div. Vet. Med., Walter Reed Army Med. Cen., Washington, D.C.
L. pomona	Sippel	Div. Vet. Med., Walter Reed Army Med. Cen., Washington, D.C.
L. pomona	Australia C	Dr. J. A. Howarth, University of California, Davis.
L. pomona	T-262	Sharp and Dohme Co., Philadelphia, Pa.
L. pomona	S-91	Mrs. M. Galton, Commun. Dis. Cen., Chamblee, Ga.
L. pomona	DM-2	Field isolate, Animal Disease and Parasite Research Division, Animal Disease Station, Beltsville, Md.
L. pomona	DM-4	Field isolate, Animal Disease and Parasite Research Division, Animal Disease Station, Beltsville, Md.
L. pomona	DM-10	Field isolate, Animal Disease and Parasite Research Division, Animal Disease Station, Beltsville, Md.

parative studies with 11 *L. pomona* strains have produced equivalent results. Cultures used in this study and their source are listed (table 1). The stock cultures were maintained in Chang's semi-solid medium containing 10 per cent normal rabbit serum and were transferred at three-month intervals. This maintenance medium, consisting of bacto-tryptose, 0.8 Gm.; bacto-liver, 0.5 Gm.; Na_2HPO_4, 2.0 Gm.; KH_2PO_4 0.4 Gm.; NaCl, 3.0 Gm.; and bacto-agar, 2.0 Gm., was adjusted to a final volume of 1,000 ml. with distilled water. The pH values of these separately stored solutions were approximately 4.50 and 7.55, respectively. The mixture of 50 ml. of the 20x concentrated solution of salts and 40 ml. of the 2x buffer was diluted to a volume of 1,000 ml. After sterilization of the basal salts at 121 C. for 15 minutes (15-lb. pressure), 7 per cent rabbit serum was added to complete the medium. This serum had the following characteristics: pH of 8.1; optical densities of 0.85, 0.10, and 0.10 at 416, 540, and 580 mμ, respectively (at 7% medium concentration); 191.0

TABLE 2—Nephelometric Growth Response of Leptospira Pomona in Different Culture Units

	Growth response					
	20 x 150-mm. tubes		250-ml. flasks		300-ml. nephelo-culture flasks	
Time (days)	Sidearm cuvette	Coleman cuvette	Sidearm cuvette	Coleman cuvette	Sidearm cuvette	Coleman cuvette
0	7.0	7.0	7.0	7.0	8.0	9.0
1	14.0	14.0	14.0	14.0	14.5	14.9
2	29.0	29.0	29.0	29.0	29.0	29.0
3	33.5	33.5	34.0	34.0	34.0	34.0
4	38.5	38.5	38.0	38.0	38.0	37.0
5	43.0	43.0	44.0	44.0	44.0	44.0
6	47.5	47.5	49.0	49.0	49.0	49.0
7	52.5	52.5	51.0	51.0	54.5	54.5
8	56.5	56.5	52.0	52.0	56.5	56.5
9	61.0	61.0	58.0	58.0	59.0	59.0
10	62.0	62.0	59.0	59.0	62.0	61.0
11	62.0	62.0	60.0	60.0	61.0	58.0

The bacto-liver was suspended separately, filtered while hot, and then added to the other components of the medium. Sterilization was carried out in screw-capped vials at 121 C. for 20 minutes at 15 lb. of pressure. When the medium cooled to 45 C., rabbit serum was added in the ratio of 1 ml. of serum to 9 ml. of medium.

Stuart's modified medium[12] with 7 per cent rabbit serum was used for growth studies. Every attempt was made throughout this investigation to maintain control over the source of serum enrichment and to screen the serum for reproducible nutritional value. Stuart's modified medium compounded in a 20x concentrated solution was composed of NaCl, 38.5 Gm.; NH_4Cl, 5.35 Gm.; $MgCl_2 \cdot 6H_2O$, 3.81 Gm.; asparagine, 2.64 Gm.; and thiamine-HCl, 0.4 Gm. The 2x concentrated phosphate buffer solution was composed of 16.6 Gm. of Na_2HPO_4 and 2.172 Gm. of KH_2PO_4.

mg. of total carbohydrate per 100 ml.; 9.15 mg. of protein nitrogen per milliliter; 55.7 per cent albumin; and 2.6 per cent *alpha*$_1$, 9.6 per cent *alpha*$_2$, 15.7 per cent *beta*, and 16.4 per cent *gamma* globulins. Protein nitrogen was estimated by the biuret test described by Gornall *et al.*,[7] using Armour's liquid protein standard (10.0 mg. protein nitrogen per ml.). The percentages of albumin and globulin were determined by paper-strip electrophoresis. The ionic strength of the buffer was 0.075, with a pH of 8.6. This procedure was carried out for a period of 16 hours at 5 ma. with a volume of 0.01 ml. of serum per strip. Total carbohydrate was measured with anthrone reagent using the method of Morris.[11]

The growth pattern of *L. pomona* was followed by employing a 300-ml. nephelo-culture flask (# 10-519) with a 19- by 130-mm. sidearm

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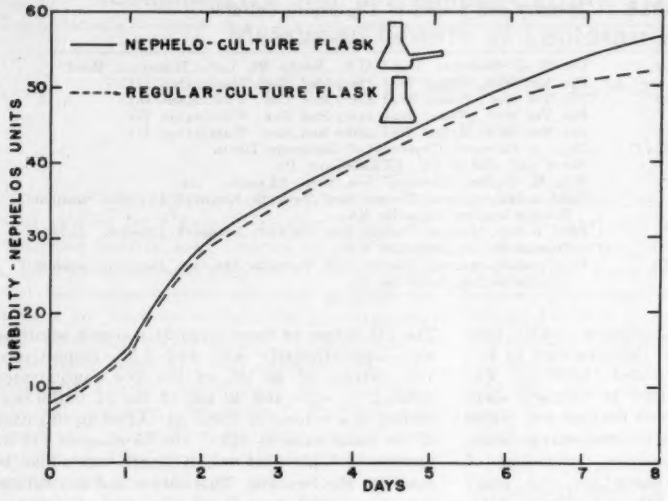

Fig. 1—The growth response of Leptospira pomona in nephelo-culture flasks and regular-culture flasks.

cuvette.* Comparative studies were carried out with nephelo-culture flasks, 250-ml. Erlenmeyer flasks, and 20- by 150-mm. test tubes. The culture turbidity was estimated by a Coleman-7 nephelometer using sidearm and Coleman cuvettes.† The instrument was adjusted to a scale reading of 60 by the direct method, employing as the biological nephelometric standard *Brucella abortus* 1119-3 tube antigen. The standard was prepared by diluting the concentrated antigen to a light transmittancy of 41 per cent at 420 mμ. The diluted standard contained approximately 1 part of antigen to 99 parts of 0.5 per cent phenolized saline solution. This product is extremely stable when protected from undue exposure to light and stored at 4–7 C.

Incubation was carried out at 28.5 to 29.0 C. in a Hotpack refrigerated incubator.* Culture flasks containing 93 ml. of Stuart's medium (after sterilization) and 7 ml. of rabbit serum were inoculated with 10 ml. from a 72-hour, 25- to 30-nephelos density culture of *L. pomona*. Culture tubes con-

* Obtained from Bellco Glass, Inc., Vineland, N.J.

† Manufactured by Coleman Instruments, Inc., Maywood, Ill.

* Manufactured by the Electric Hotpack Co., Inc., Philadelphia, Pa.

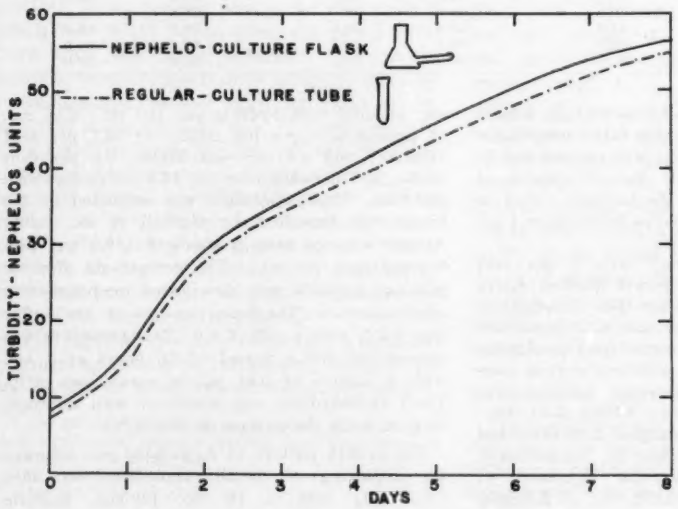

Fig. 2—The growth response of Leptospira pomona in nephelo-culture flasks and regular-culture tubes.

taining 9.3 ml. of medium and 0.7 ml. of rabbit serum received 1 ml. of the same inoculum. Growth response in nephelos units was plotted on equal division graph paper on the vertical axis against incubation time in days on the horizontal axis.

RESULTS

The growth response studies carried out with 300-ml. nephelo-culture flasks, 250-ml. Erlenmeyer flasks, and 20- by 150-mm. test tubes compared favorably with one another quantitatively regardless of the cuvette employed. The Pyrex sidearm cuvettes of the nephelo-culture flasks have optical properties very similar to those of the conventional Coleman cuvettes. This is demonstrated by the results (table 2 and fig. 1–3).

The growth response of *L. pomona* in Erlenmeyer flasks and nephelo-culture flasks is presented (fig. 1). In order to show these data, it was necessary to plot the nephelos values of growth in regular flasks 1 unit below those in the nephelo-culture flasks from day 0 through day 5. Comparisons (table 2) show that the values actually are identical. The slight variation observed at day 0 is attributable to manipulative procedures, since the same density and age of inoculum were used throughout the study. A comparison between a test tube assay done in 1955 and a nephelo-culture flask assay carried out in 1958 is illustrated (fig. 2). This pattern of reproducibility has been continually possible throughout this and other studies of nutritional and culture characteristics of *L. pomona*.

A comparison of three individual leptospires isolated from three different bovine hosts is shown (fig. 3). These isolates were made from the same animal herd under identical conditions and with identical procedures. Prior to serological typing, the cultures (designated DM-2, DM-4, and DM-10) were characterized as to their growth response using nephelo-culture flasks. The minute variation in the quantitative growth response is due to minor variation in inoculation density. The pattern of growth shown in figure 3 is quantitatively different from that shown in figures 1 and 2. It is to be emphasized that the culture response shown in figures 1 and 2 represents the growth curves of a long-time laboratory strain, whereas in figure 3 the cultures employed represent recently isolated field strains. Prior to growth assay, these field strains had been adapted to maximum growth response in Stuart's medium. Subsequent serotyping proved these three isolates to be *L. pomona* and to be identical in their cross-agglutination pattern.

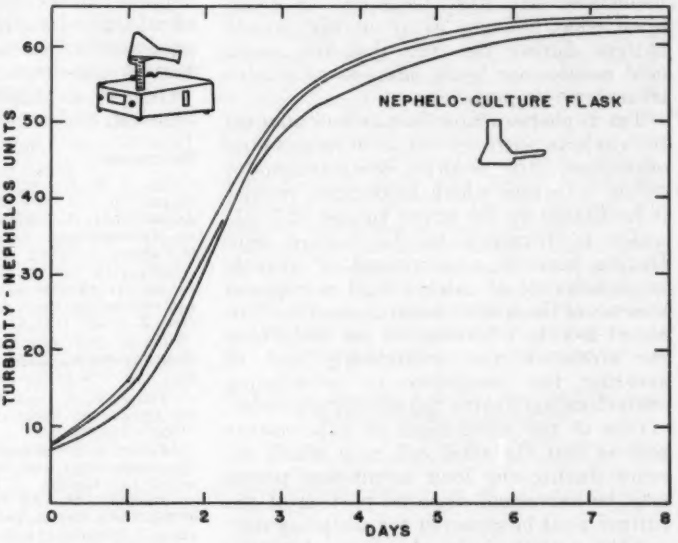

Fig. 3—The growth response of three Leptospira isolates from separate bovine hosts, located in one animal herd. These three strains proved to be Leptospira pomona.

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DISCUSSION

The results of the growth studies performed with *L. pomona* using nephelo-culture flasks show the data are similar to those obtained by more tedious methods. The sharp differences seen in the growth rates of the three field isolates versus long-time laboratory cultures cannot at this time be completely explained. The variability in growth rate is probably characteristic of different strains and not attributable to a history of recent isolation. These differences should be further studied, using the nephelo-culture flask technique to determine what changes occur in the growth pattern during the time that the recent field isolates are being adapted to routine laboratory propagation.

The nephelo-culture flask is well adapted to various studies with slow-growing organisms. The accurate measurement of serum substrate which leptospires require is facilitated by the larger volume of 7 ml., which is dispensed to the culture unit. During periodic measurement of growth, no withdrawal of culture fluid is required because of the useful sidearm cuvette. This aspect has the advantage of not disturbing the culture-volume relationship and of avoiding the possibility of introducing contamination during the sampling process.

One of the advantages of this culture unit is that the total cell crop which accrues during the long incubation period may be harvested, since no portion of the culture need be removed for sampling during the growth cycle. Another advantage of the nephelo-culture flask is the safety in handling highly virulent bacteria. This method of culture would possibly obviate some of the manipulations resorted to by Fisher and Kirchheimer [4] in their nephelometric studies of *Mycobacterium tuberculosis*.

The preparation of inoculums to exact nephelos-densities is greatly facilitated with nephelo-culture flasks. Adjustment to specific turbidimeteric values is possible by dilution with sterile modified Stuart's medium. Samples do not have to be withdrawn, and a close check of nephelos values can be made simply and rapidly with the sidearm cuvette. Antigen densities for standardized agglutination-lysis tests can be carefully estimated and adjusted. The addition of enrichments or inhibitors to mediums and their effect on growth can be assessed as long as the volume incorporated is not appreciable.

SUMMARY

The nephelometric growth response of *Leptospira pomona* has been studied employing a new, commercially available nephelo-culture flask. This unit offers the advantage of rapidity, simplicity, and reproducibility, as well as the opportunity to harvest cell crops which can then be subjected to a variety of biochemical, biophysical, and serological studies.

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The Identification of Viable and Nonviable *Erysipelothrix Insidiosa* with Fluorescent Antibody

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ERYSIPELOTHRIX INSIDIOSA has been incriminated as the causative agent in diseases of animals, fish, fowl, and human beings. While it is recognized that at least four distinct serologic types exist, all share common major antigenic factors.[2,8] Isolation of *Ery. insidiosa* from infected material and its subsequent identification is time-consuming; this is particularly true when the original specimen is grossly contaminated with heterologous species of bacteria.

The fluorescent antibody (FA) technique has been used to advantage in the rapid identification of microorganisms.[3,7,9] It has the further advantage of being useful in the identification of nonviable antigens.[1,4]

This paper reports the application of the FA technique to the identification of *Ery. insidiosa* in pure and mixed cultures and in fixed tissues.

MATERIALS AND METHODS

The *Ery. insidiosa* ARS standard antiserum (lot 10, equine origin) * was conjugated with fluorescein isothiocyanate.[5] Normal rabbit serum and antiserums prepared against *Listeria monocytogenes*, types I–IV, were conjugated at the same time and used as control serums. A bovine serum was conjugated with rhodamine † and used in a proportion of 1 part to 20 of fluorescein-conjugated serum as a counterstain.[6]

Strains Used.—All strains of bacteria used in these experiments were maintained on trypticase soy agar slants that contained yeast extract. Strains of *Ery. insidiosa* used in this study were obtained as follows: Seventeen strains of swine or turkey origin from Dr. B. B. Hancock, Ohio State University, Columbus; four strains of swine origin, from Dr. R. Shuman, U.S.D.A., Beltsville, Md.; and seven strains of swine or human origin, from the Walter Reed Army Institute of Research, Washington, D.C. Heterologous species were taken from stock cultures maintained for other projects.

Ultraviolet Microscope.—A Zeiss microscope equipped with neofluar objectives was used in conjunction with an OSRAM HBO 200 high-intensity light source. Two filter combinations were used routinely, a BG12 exciter filter with a GG4 and an OG4 barrier filter combination, and a UG5 exciter filter with the GG4 barrier filter.

Procedure for Categorizing the Specificity of Reactions.—Smears were prepared from saline suspensions of pure cultures of 28 strains of *Ery. insidiosa* and 150 strains of heterologous bacteria. The smears were air dried, heat fixed, and stained by overlaying with conjugated serum for 15 minutes at room temperature in a moist chamber. After staining, slides were washed in buffered saline solution (pH 7.2) for 10 minutes and mounted in buffered glycerine (pH 7.2).

Procedures for Mixed Cultures and Clinical Material.—Suspensions in broth were prepared from cultures that contained various combinations of *Ery. insidiosa* with Proteus sp., Pseudomonas sp., Bacillus sp., *Escherichia coli*, *Staphylococcus aureus*, and Streptococcus sp., so that the mixture contained approximately 3×10^9 organisms per cubic centimeter. *Erysipelothrix insidiosa* was present in the mixture in proportions of from $1:10^2$ to $1:10^6$.* After smears were prepared, the remainder of the sample was subjected to routine bacteriologic analysis. Clinical material was obtained from two pigs suspected of having erysipelas and from experimentally infected mice and pigeons.

Procedure for Testing Stability of the Antigen.—Smears of the 28 strains of *Ery. insidiosa* were prepared, air dried, and each exposed to one of the following fixatives: 10 per cent formalin, 18

TABLE 1—Specificity of Straining with Erysipelothrix Insidiosa Antiserum

Organism	No. of strains	No. positive
Erysipelothrix insidiosa	28	28
Listeria monocytogenes	25	0
Salmonella sp.	30	0
Shigella sp.	10	0
Proteus sp.	10	0
Pasteurella sp.	30	0
Brucella sp.	6	0
Tribe Mimae	17	1
Neisseria sp.	7	0
Escherichia sp.	15	0

From the Armed Forces Institute of Pathology, Washington, D.C.

* Obtained from Dr. Richard Shuman, Animal Disease Research Center, U.S.D.A., Beltsville, Md.

† Lissamine rhodamine RB200, produced by Imperial Chemical Industries, Ltd., London, England.

* Suspensions of a concentration of $1:10^2$ contained approximately 3.0×10^7 *Ery. insidiosa* and 2.97×10^9 heterologous organisms per cubic centimeter. The $1:10^6$ concentration contained 3.0×10^3 *Ery. insidiosa* and 3.0×10^9 heterologous organisms per cubic centimeter.

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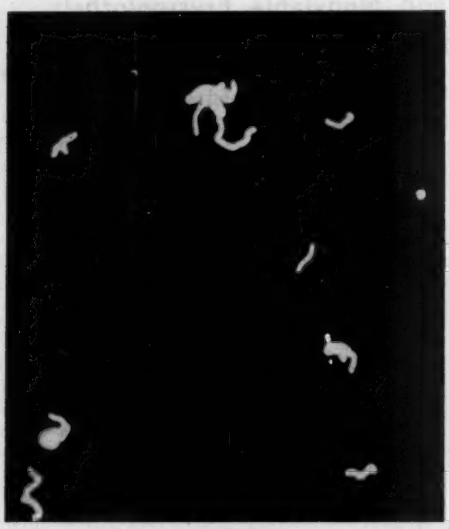

Fig. 1—Erysipelothrix insidiosa isolated from blood of a human being with septicemia and stained by the fluorescent antibody technique. x 500.

hours; 95 per cent ethanol, one hour; absolute methanol, one hour; acetone, one hour, Zenker's solution, one hour; or Bouin's solution, one hour. A set of slides was also prepared from distilled water suspensions that had been boiled for one hour, air dried, and heat fixed. All chemically fixed smears were washed in repeated changes of buffered saline solution to remove excess fixative, air dried, and stained as above.

Procedure for Tissues.—Mice were infected by intravenous inoculation of 0.1 ml. of a saline suspension from a 24-hour slant of *Ery. insidiosa*. Pigeons were inoculated intramuscularly with 0.2 ml. of a similar suspension. At necropsy, sections of muscle, spleen, liver, heart, lung, and kidney were placed in 10 per cent neutral formalin. Sections were trimmed after fixation (7–10 days), carried through the standard embedding process, and cut at 5 to 7 μ. The sections were deparaffinized through two changes of xylene and two of graded alcohol. The sections were then washed for ten minutes each in at least two changes of buffered saline solution to remove all traces of alcohol and then stained in the routine method.

RESULTS

Categorization of the Specific Antiserum.—Positive staining was observed with all 28 strains of *Ery. insidiosa* tested with the homologous serum (fig. 1, 2). When the serum was tested against 150 strains of heterologous organisms, 149 strains were negative and a single strain of *Bacterium anitratum* showed a weak staining reaction. The bacteria tested and the results of staining with the specific *Ery. insidiosa* antiserum are listed (table 1). When the 28 strains of *Ery. insidiosa* were stained with conjugated pooled "normal" rabbit serum or serums prepared against *Listeria monocytogenes*, types I–IV, no staining occurred.

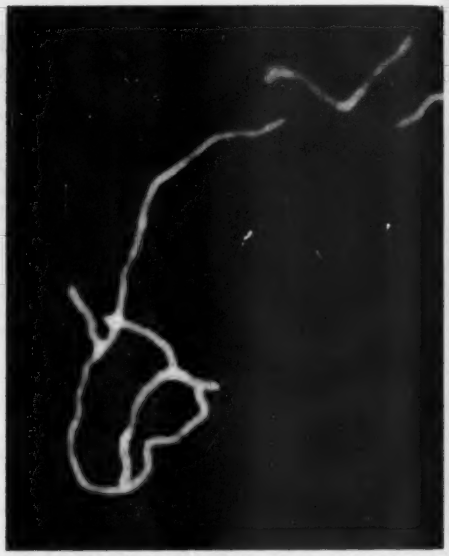

Fig. 2—Erysipelothrix insidiosa, rough stock culture. Notice apparent branching. x 500.

Mixed Cultures and Clinical Material.—When smears from mixed cultures were examined, those containing *Ery. insidiosa* in preparations greater than $1:10^3$ required observation of from three to ten high-power fields for identification of the organism. Those smears containing fewer *Ery. insidiosa* organisms in the total population required more study. When the entire smear was observed, identification was possible if the order of incidence was as small as $1:10^6$. The average time necessary for identification by routine culture methods was three to 12 days, depending on relative numbers and co-contaminants.

The FA technique permitted rapid recognition of *Ery. insidiosa* when present in clinical material consisting of blood smears, exudate, and impression smears. The most striking examples were blood smears from 2 pigs with septicemia. Organisms were

observed both in phagocytic cells and in serum.

Stability of Antigen.—The staining properties of all 28 strains of *Ery. insidiosa* were not affected in any detectable manner by exposure to the fixative used. Boiling for one hour prior to the preparation of smears had no deleterious effect on the staining reaction. It was postulated that if the antigenic components against which the serum was prepared could withstand these various treatments, then the processes of fixation, embedding, and deparaffinizing should not alter them.

Erysipelothrix Insidiosa in Tissue Sections.—Sections from the spleen, liver, and kidney of infected mice showed areas of microscopic necrosis that, when stained with Giemsa's or Brown and Brenn's stains, appeared to be filled with organisms. When sections were stained with conjugated anti-*Ery. insidiosa* serum, the organisms were observed as bright green masses in a red-orange cellular background under low power (fig. 3). When viewed at a magnification of x 400, individual organisms were distinguishable. Control sections stained with heterologous or normal serum appeared uniformly orange-red throughout (fig. 4). Sections of lung contained massive areas of bacterial growth that were identified as *Ery. insidiosa* by the FA technique. Sections of heart showed a few organisms in the interstitial spaces, but no evidence of vegetative endocarditis was present.

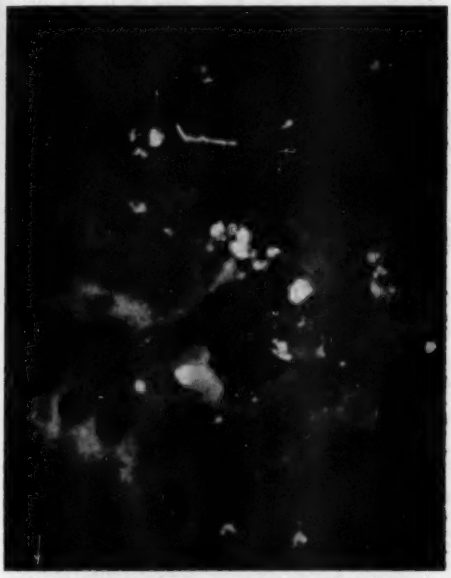

Fig. 3—Formalin-fixed, paraffin-embedded liver section from a mouse infected with Erysipelothrix insidiosa stained with specific antiserum. Individual organisms are visible. x 125.

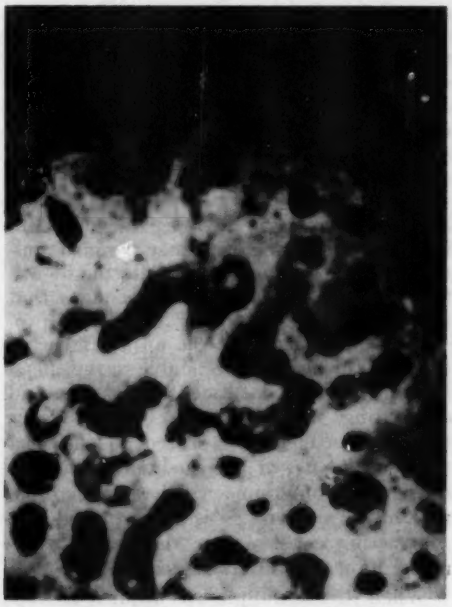

Fig. 4—Serial section of liver stained with normal rabbit serum. No organisms seen. x 125.

Sections from the pigeons, while qualitatively similar to those of the mice, did not show the massive involvement of the organs. The only tissue showing comparable bacterial growth was a section of muscle at the site of inoculation.

Tissues that had appeared devoid of organisms when stained with routine aniline dyes were observed to contain a small number of organisms occurring singly throughout the section when stained by the FA technique.

When comparable tissues from animals infected with other diseases were stained with *Ery. insidiosa* antiserum, no specific green fluorescence was observed.

Discussion

The fluorescent antibody technique is a useful tool in the identification of micro-

organisms when adequate controls are incorporated. The importance of categorizing the reactivity of the serum used cannot be overemphasized. If the serum used is specific (does not react with commonly occurring heterologous organisms) and contains antibody to at least one antigenic fraction common to all strains of the organism for which it was prepared, it can facilitate the identification of the microorganism in clinical specimens or in primary mixed cultures. The FA technique permits the identification of the organism in specimens that are no longer suitable for culture procedures, provided that the antigenic properties of the bacteria are stable to the chemical and physical treatment of fixation and embedding. An additional advantage is that the antigen is localized in the host tissues. The use of the rhodamine counterstain in conjunction with the specific fluorescein-conjugated antiserum provides a contrasting color scheme that permits the recognition of a single bacterium.

When this technique was applied to *Ery. insidiosa*, the time necessary for its identification was greatly reduced in comparison with standard methods. Specimens containing as few as 1 in 10^3 *Ery. insidiosa* required less than one hour for identification. In tissues, the relative distribution of *Ery. insidiosa* was observed; due to the specificity of the reaction, individual organisms could be identified with a high degree of certainty.

The relative stability of the antigenic components of *Ery. insidiosa* should permit the examination of material that has been obtained in prior experiments and processed for histologic studies.

SUMMARY

The fluorescent antibody technique as applied to *Erysipelothrix insidiosa* was investigated. The specificity of reaction was such that all 28 homologous strains tested were positively stained, while there was no reaction in 149 of 150 heterologous strains. The exception was a weak staining reaction of one strain of *Bacterium anitratum*. Chemical and physical treatments involved in histologic preparation of tissue specimens did not destroy the staining reaction. *Erysipelothrix insidiosa* was demonstrated in formalin-fixed, paraffin-embedded tissues.

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Hematological Effects of Acute and Chronic Experimental Blood Loss in the *Macaca Mulatta* Monkey

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PRIOR to the planned utilization of the *Macaca mulatta* monkey in an extensive radiobiological study, which was to include examination of hematological effects, a search of the relevant literature was carried out. It revealed no detailed account of the effects of either acute or chronic blood loss in this animal. Because it was anticipated that experimental procedures and sampling would produce a certain inevitable loss of blood, several series of baseline studies of this factor were performed. In view of the generally increased interest in the monkey as an experimental animal, the results of these preliminary studies are reported herein.

MATERIALS AND METHODS

Thirty male *Macaca mulatta* monkeys, approximately 36 months old and weighing 1.8 to 3.6 kg., were studied. Assuming a blood volume–body weight ratio of 60.9 ml. per kilogram,[2] the average blood volume of these animals was approximately 164 ml. All animals were maintained on a standard diet,[20] and blood samples were never obtained earlier than 15 hours after feeding.

The 30 animals were allotted at random to six groups of 5 animals each as follows: Control group—4 ml. of blood (about 2.5% of blood volume) removed per week for hematological determinations; acute group—30 ml. removed initially and 30 ml. 80 minutes later, totaling 60 ml. (about 37%); chronic group 1—10 ml. removed (about 6%); chronic group 2—20 ml. removed (about 12%); chronic group 3—30 ml. removed (about 18%); and chronic group 4—40 ml. removed (about 24%).

Hematological observations were made in the acute group at one, two, three, and four days, then once a week for the next six weeks following experimental blood loss. Each observation required 3 to 4 ml. of blood. Blood samples were taken from monkeys in all chronic groups weekly for eight weeks. Blood from animals in chronic and control groups was studied hematologically at those times.

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The following hematological analyses were carried out using techniques previously described:[13] erythrocyte count and hemoglobin, reticulocyte, and hematocrit evaluations. Blood samples were obtained by puncture of the femoral artery or by venipuncture. Counts were performed on a heparinized aliquot of the blood thus obtained. The data were submitted to statistical analysis by the two-way analysis of variance* in which a p value of 0.05 or less was regarded as significant.

RESULTS

Acute Blood Loss.—Within four days following the removal of two 30-ml. aliquots of blood (approx. 37% of the circulating blood volume) the average erythrocyte count fell from 4.76×10^6 cells per cubic millimeter to a low of 4.08×10^6 cells per cubic millimeter. The decline was significant ($p<0.005$). Between the fourth and seventh day the count began to rise, reaching a peak by the fourteenth day, subsequently declining, then rising again by the twenty eighth day to a plateau which was maintained until the end of the experiment. It was considered that the erythrocyte count had returned to the normal range by the twenty-eighth day, as there was no significant difference between the counts in the experimental and control animals at that point (fig. 1).

As might be expected, the hemoglobin change paralleled that of the erythrocyte count, reaching a maximum depression on about the second day, rebounding temporarily by the fourteenth day, and returning to normal values by the twenty-eighth day (fig. 2).

The response of reticulocytes to the loss of over a third of the blood volume was dramatic. Between the first and third day there was an apparent increase reaching a peak of 4.4 per cent on the fourth day followed by a decline to within the normal range by day 14 (fig. 3).

Chronic Blood Loss.—The repeated loss of blood resulted in prompt decrease in the

* The statistical treatment of this data was prepared by Mr. David Rubinstein, Department of Biometrics, School of Aviation Medicine, USAF, Randolph Air Force Base, Texas, whose help is gratefully acknowledged.

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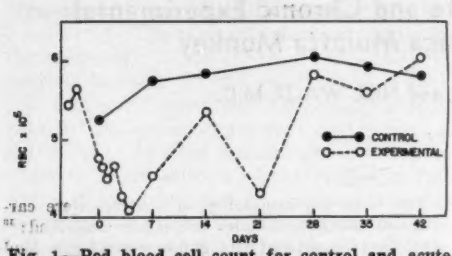

Fig. 1—Red blood cell count for control and acute blood loss groups (37% blood loss). Each point represent the average of five individual values.

erythrocyte count (fig. 4). The extent and persistence of the depression appeared generally related to the amount of blood loss. In all experimental animal groups the average count was below the average for the control animals. Also, the counts generally fell below the individual group baseline levels. This was least pronounced in the monkeys with a loss of only 10 ml. per week (group 1). The animals undergoing a loss of 40 ml. per week (group 4) showed a temporary rebound toward normal by the fourth week but, at the termination of the experiment, fell back to a plateau significantly below its own baseline and below the level of the other groups, including the control group. A similar and better-maintained rebound occurred in the animals with a smaller blood loss as well, with the control group (4-ml. loss per week) showing an actual increase.

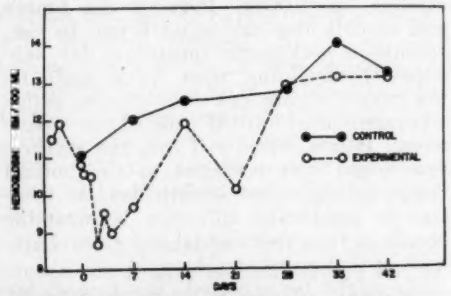

Fig. 2—Hemoglobin (gm./100 ml.) values for control and acute blood loss groups (37% blood loss). Each point represents the average of five individual values.

The hemoglobin response to experimental blood loss (fig. 5) was much like that of the erythrocyte count. Here again, the animals with a smaller blood loss seemed to recover during the test period, with the hemoglobin values returning to group baseline levels or above by the end of the study (fig. 5). Again, the control group showed an actual increase in hemoglobin.

Reticulocyte response (fig. 6) in the monkey was seen to be varied, with the exception of the animals in chronic group 4 (loss of 40 ml. per week). In this group

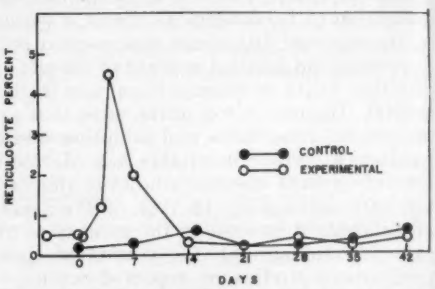

Fig. 3—Reticulocyte values (% r.b.c.) for control and acute blood loss groups (37% blood loss). Each point represents the average of five individuals values.

there was a rather prompt response, reaching an initial peak at the third week and followed by another peak of reticulocytosis by the fifth week. This was followed by a sharp reduction in reticulocytes to normal limits at the eighth week. In the animals in chronic group 1 (10-ml. loss) there appeared to be a slight increase in reticulocytes at the fifth week, but this trend is not significantly different from the controls.

The hematocrit values for all experimental groups fell during the period of observation. The only animals demonstrating any recovery in hematocrit level were those in group 2 (20-ml. loss). The decreases in hematocrit level did not correlate well with amounts of blood lost. Large individual variations detract from the significance of the rather small group differences. Thus, none of the changes in hematocrit level are statistically significant when tested by the analysis of variance technique.

DISCUSSION

From the results of acute blood-loss studies in the monkeys reported here, it appears that these animals can withstand an abrupt loss of about a third of the average circulating blood volume without irreversible ill effects and with apparent return to

hematological normalcy within 28 days. The response of the marrow is demonstrated by the prompt increase in circulating reticulocytes which reach a peak at four days after blood loss. These results are in accord with similar findings in other species.

Jencks,[11] studying blood regeneration in the albino rat, found that a blood loss of 33.5 per cent could be tolerated with an average recovery time, as measured by hemoglobin concentration, of about seven days. Davis *et al.*[6] found that within 48 hours following removal of 50.0 per cent of the blood volume of the albino rat, there was an immediate and approximately sixfold increase in circulating reticulocytes and full recovery of erythrocyte volume in seven days.

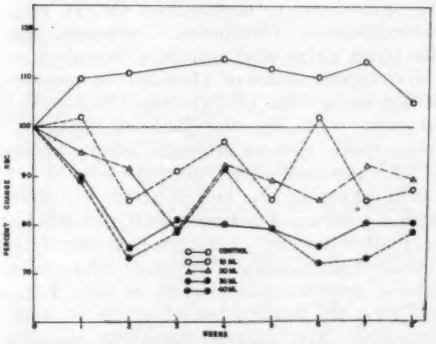

Fig. 4—Percentage of change in red blood cell count from individual group baseline values for control and chronic blood loss animals 10-, 20-, 30-, and 40-ml. loss a week for eight weeks). Each point represents the average of five individual values.

Martin and Myers[14] studied the effects of a 500-ml. blood donation on the human donor and found a 24-hour increase in reticulocytes up to 11 per cent. They report that only six days were required for the erythrocyte counts to return to normal. Fowler and Barer,[8] however, reporting on human blood donors, state that the average time required to replace an average 2.3-Gm. fall in hemoglobin, following a 555-ml. blood loss, was 49.6 days, with a range of 18 to 98 days among 155 subjects adequately studied.

In the *Macaca mulatta* monkey, under the environmental conditions of our colony, response to acute blood loss of 37 per cent of the blood volume appears to be in qualitative accord with the response of other species, with regeneration occurring in about a month.

Analysis of the hematological results of weekly blood loss in the young male monkeys utilized in this study concerning chronic blood loss suggests that there is either little or no significant alteration of the peripheral erythrocytes following a blood loss of 4 ml. (controls) or 10 ml. per week for eight weeks. These results agree with the findings of Chadham,[4] studying 50 human donors who had recovered from poliomyelitis and were giving 65 to 140 ml. of blood at weekly intervals for six to 12 weeks. It is evident that the ability of the bone marrow to maintain an adequate compensatory rate of replacement of the peripheral blood lost was sufficient to prevent anemia at these levels of blood loss.

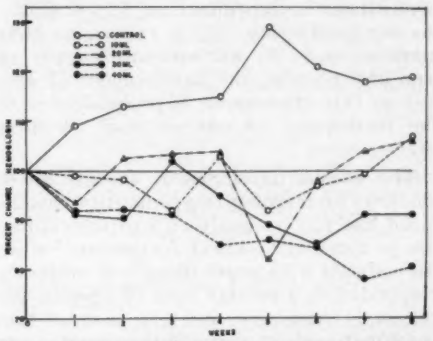

Fig. 5—Percentage of change in average hemoglobin values from individual group baseline values for control and chronic blood loss groups.

However, the results of weekly blood losses of 20 ml. (12% of the blood volume), 30 ml. (18%), and 40 ml. (24%) indicate

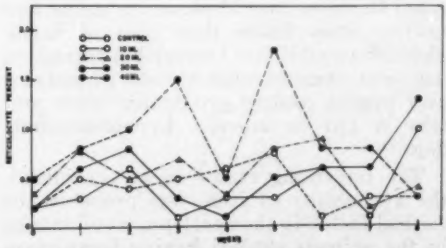

Fig. 6—Average reticulocyte values (% r.b.c.) for control and chronic blood loss groups (10-, 20-, 30-, and 40-ml. loss per week for eight weeks). Each point represents the average of five individual values.

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that compensatory bone marrow erythropoiesis could not keep pace with the greater repeated loss. The decrease in erythrocyte count, hematocrit reading, and hemoglobin are indicative of this decompensation.

Since the maintenance of erythrocytic homeostasis is dependent on (1) a stimulus for production, (2) a responsive bone marrow, and (3) an adequate supply of iron and protein, the development of anemia in this experiment is probably due to the inadequacy of one or more of these prerequisites.

The anemia developed by the groups of monkeys undergoing large-quantity chronic blood loss was probably an adequate stimulus to accelerated blood formation,[16] since the animals with acute blood loss evidently responded to a similar level of anemia. By the same reasoning, one can assume that the functional integrity of the marrow was unimpaired and that it was able to respond if given the necessary materials. The reticulocyte increases in the animals with acute and large-quantity chronic blood loss are also indicative of marrow response, although no quantitative correlations should be drawn.[19]

It has been observed that protein depletion probably plays a part in the production of anemia in malnourished chronic blood donors.[1] Also, in nonanemic rats, protein depletion alone causes a marked decline in rate of erythropoiesis,[3] perhaps accentuated by the absence of the stimulatory effect of anemia on blood production.[10] However, in this study, a significant etiological role for protein depletion is unlikely, since rapid plasma regeneration has been shown to occur in man and other mammals on a normal diet, as our subjects presumably were. This is attributed to reserve stores of available protein as well as to the rapid turnover rate of plasma proteins, five to ten times faster than that of hemoglobin.[5] In addition, hemoglobin formation has been demonstrated to take precedence over plasma protein production when protein is fed to anemic, hypoproteinemic dogs.[18]

The remaining factor to be considered, the availability of iron, was probably the critical factor in the development of anemia in the animals studied having large-quantity chronic blood loss. Measurements of blood regeneration after acute and chronic blood loss in various animals and in man have shown great variations in recovery time, ranging from a few days to months.[7, 9, 12, 17] However, when the availability of iron has been measured in such experiments, it has become clear that the rate of blood regeneration is proportional to the amount of available iron, whether in body stores or ingested.[5] Therefore, it can be concluded that the development of anemia in the animals losing the larger amounts of blood, and in particular the iron component of that blood, reflects the depletion of body iron stores, together with the inadequacy of the normal dietary intake to compensate for the amount lost.

Although measurement of iron metabolism was not performed in this study, certain related facts can be given. The work of Hartwig *et al.*,[10] using other monkeys of the same colony, maintained in the same environmental conditions, indicates that the mean value and standard deviation of 455 determinations of plasma iron sampled at the same time of day was 185.3 ± 46.0 μg. per cent, by the Ramsey method.[15] Also, there was an average incorporation of 10.7 per cent of a 5-μc. injection of ferric-59 chloride 24 hours after injection, with a plateau of between 90.0 and 100 per cent after a week. A rough analysis of the standard diet in use[20] gives an average intake of approximately 54 to 70 mg. of iron per day, the adsorption of which is undetermined. Approximate total iron loss over the experimental period in our monkeys was as follows: acute group, 37 mg.; chronic control group, 14 mg.; chronic 10-ml. loss group, 32 mg.; chronic 20-ml. loss group, 54 mg.; chronic 30-ml. loss group, 85 mg.; and chronic 40-ml. loss group, 98 mg.

The monkey colony used in this study is maintained in a standardized, controlled environment, which includes a carefully regulated diet.[20] Therefore, the empirical determination of permissible experimental blood loss by the means reported herein is considered an adequate guide to the planning of hematological sampling in the design of other experiments utilizing *Macaca mulatta* monkeys, similarly maintained, over similar experimental periods.

SUMMARY

1) Thirty normal male *Macaca mulatta* monkeys, weighing 1.8 to 3.6 kg., were allotted to six groups of 5 animals each and subjected, respectively, to acute blood loss

of 60 ml. (37.0% of blood volume), or to chronic blood loss of 4 ml. (2.5%), 10 ml. (6.0%), 20 ml. (12.0%), 30 ml. (18.0%), or 40 ml. (24.0%) weekly for a period of eight weeks.

2) The animals which underwent severe acute blood loss demonstrated a return of the erythroid elements of the peripheral blood to normal within 28 days.

3) In animals with chronic experimental blood loss, weekly loss of 4 or 10 ml. did not alter the peripheral erythroid elements significantly, whereas 20-, 30-, and 40-ml. losses could not be compensated for during the experimental period of eight weeks.

4) The results and implications of the acute and chronic blood loss experiments are compared with those obtained in other species, including man.

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The Digestibility of Animal Fats and Proteins by Mink

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THE GROWTH, fur production, and reproductive performance of mink is dependent upon the availability of the nutrients in the diet as well as on the nutritional content of the diet presented to the animal. Although considerable information is available on the proximate analysis of mink feed products, few data are available on the digestibility of these products for mink. Bernard *et al.*[4] were the first workers to report studies on the digestibility of feed products by mink. Their experiments were concerned with the digestibility of fish and plant products by mink. Although some experimental data were presented on the digestibility of fish fat and protein, the study was primarily related to the digestibility of cereal carbohydrates.

The economic production of quality mink pelts will be facilitated by a knowledge of the digestibility, for mink, of the animal and fish products used on commercial mink ranches. The present study has been undertaken to ascertain the digestibility of animal fats and proteins by mink.

EXPERIMENTAL PROCEDURE

Diet Composition.—The composition of the diets used in the digestion trials is shown (tables 1, 2). These diets were formulated on the basis of current ranch practice. The feed products chosen for study are those in common use on American mink ranches.

For the study on the digestibility of fats, two basal diets were employed. Basal diet 2 was used for the tests on the digestibility of the fats present in horse meat, beef, and poultry by-products. Ground oat groats, wheat bran, and fish flour were selected on the basis of palatability (for mink) and low fat content. The fish flour product contained less than 0.1 per cent fat. The wheat bran was added at a level which provided a dietary fiber content similar to that present in mink diets used commercially. The chicken by-product mixture used in this study contained approximately 50.0 per cent entrails, 25.0 per cent heads, and 25.0 per cent feet. On fat-free diets, significant quantities of fat, originating primarily from digestive processes, are found in the feces. The amount of this fecal fat, termed "metabolic fat," must be taken into account for an accurate determination of the true digestibility of fats. In this study, a low-fat diet was employed to ascertain the metabolic fat exeretion of mink on the test diets. This basal metabolic diet was similar to basal diet 1 and contained 80.0 per cent fish (fish flour plus water) with no fat supplement. This basal metabolic diet contained 2.3 per cent fat on a dehydrated basis. The diets used in the basal diet 1 series contained approximately 22.0 per cent fat. In the basal diet 2 series, however, it was not possible to maintain a uniform fat content in the experimental diets because of variation in the fat content of the products tested. The dietary fat composition for the basal 2 series was: beef lung diet, 7.6 per cent; horse meat diet, 8.6 per cent; beef rumen diet, 27.5 per cent, and chicken by-product diet, 34.9 per cent.

TABLE 1—Composition of Basal Diets—Fat Digestibility Study

Ingredients	Basal diet (%) 1	2
Ground oat groats	16	16
Wheat bran	4	4
Fish*	72	....
Processed fat	8	....
Animal product	....	80

* Composed of one part VioBin fish flour (VioBin Corp., Monticello, Ill.) and four parts water.

For the study on the digestibility of proteins, a different type of test diet was employed. In order to limit the protein content of the test diet to one specific source, it was necessary to formulate a special dietary mixture which did not contain cereal products (table 2). The formulation used in this study provided a ration with a proximate analysis similar to that of commercial mink ranch diets. The diet was designed on the basis of maintaining the nutrient composition of all test diets as similar as possible in respect to fat, carbohydrate, protein, and fiber content. Inasmuch as the type of carbohydrate and the level of fat in the diet may influence the time of passage of feed through an animal's digestive tract, the level of these two nutrients was maintained constant in all test diets except for the chicken entrail diet which contained a higher level of fat. Vitamin fortification of the test diets was similar to that used in basic research studies with mink.[5] The protein content of the diets varied somewhat relative to the ash content of the feed product tested. The protein composition of the diets was: horse meat

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diet, 40.8 per cent; beef lung diet, 40.2 per cent; ocean whiting diet, 38.0 per cent; beef rumen diet, 37.0 per cent; chicken head diet, 32.2 per cent; chicken feet diet, 27.4 per cent; and chicken entrail diet, 15.6 per cent. The high ash content of the chicken heads and feet and the high fat composition of the chicken entrails were responsible for the lower level of protein in these test diets.

TABLE 2—Composition of Diets—Protein Digestibility Study

Ingredients	Percentage*
Protein—animal or fish plus ash	43.0
Fat—animal or fish plus lard	25.0
Carbohydrate—sucrose	25.0
Fiber—cellulose†	5.0
Minerals—$CaHPO_4$	1.0
NaCl	0.5
Vitamins**	0.5

* Percentage is expressed on a dehydrated basis. † Solka-Floc, Brown Co., Berlin, N.H. ** See text.

Feed Preparation.—All test feed products were obtained fresh from the processing plants, ground three times with a ⅜-inch grinder and thoroughly mixed. Prior to grinding, the chicken entrails were individually washed to remove foreign matter. The feed products were then analyzed for moisture and fat content. The test diets for the protein digestibility studies were prepared by adding sufficient sucrose, cellulose, minerals, vitamins, and lard (mixed with the sucrose and cellulose) to provide the desired proximate composition. The test diets for the basal diet 1 series of the fat digestibility studies were prepared by thoroughly mixing the melted fat with the cereal portion prior to the addition of the fish flour and water. This procedure was necessary to provide an even distribution of the test fat throughout the ration. To insure uniform feed composition throughout the digestion trial, enough experimental diet was mixed at one time for the entire feeding period for that diet. A representative sample was removed for chemical analysis and the remaining feed frozen in individual containers (1 day's feed portion) at −5 C. Maximum storage for the experimental diets was six days.

Feeding Program.—Ten male, pastel mink were used for each digestion trial. The mink were placed in individual metabolism cages consisting of 1 by 2 by 1½ ft. mink pens provided with stainless steel urine collection jackets. Copper wire screens were provided for the separation of urine and feces.

A seven-day experimental feeding period was used. This period consisted of a two-day preliminary feeding phase and a five-day fecal collection phase. Daily collection of waste feed and feces facilitated the differentiation of dried waste feed and mink feces and resulted in minimal contamination of the mink feces by urine. Twelve hours prior to the initiation of the fecal collection phase and 12 hours before the termination of the 120-hour period, all feed was removed from the metabolism cages. Inasmuch as passage of feed through the digestive tract of the mink is completed within 4½ hours,[5, 12] this procedure insured that the feces collected represented feed consumed during the five-day fecal collection phase. Animals were given either 300 or 350 Gm. of feed daily, corresponding to that amount of feed that would slightly exceed the demands of the mink.

Chemical Analyses.—Feces collected for the study on the digestibility of fats were frozen and stored at −5 C. until prepared for chemical analysis. Feces collected during the protein digestibility study were placed in 0.8 N H_2SO_4 prior to preparation for chemical analysis. Feed samples, waste feed, and mink feces were dried at 70 C. for one week, weighed, ground in a Wiley mill, and representative samples were taken for chemical analysis. Feed samples were analyzed for fat, protein, and moisture by standard procedures.[3] The procedure of Van de Kamer *et al.*[13] was followed for the determination of fat in the fecal samples. This method determines fat present as neutral fat, soaps, and free fatty acids. The Kjeldahl procedure[3] was used for the determination of fecal protein.

Digestibility Coefficients.—The coefficients of digestibility for fat represent true digestibility values inasmuch as corrections have been made for metabolic fat present in the feces. The corrections for metabolic fat were calculated on the basis of the total fat (metabolic and undigested dietary) excreted by the mink on the low-fat basal metabolic diet. Fat excretion of the mink on the basal metabolic diet was uniform. The total fat composition of the dehydrated feces averaged 3.4 per cent and varied from 3.3 to 3.6 per cent. The fat digestibility coefficients were calculated on the basis of the following formulas:

$$\%\ \text{indigestible fat} = \frac{(\text{Gm. feces})\,(\%\ \text{fecal fat} - 3.4\%)}{\text{Gm. fat consumed}}$$

$$\text{Coefficient of true digestibility} = 100\% - \%\ \text{indigestible fat}$$

The coefficients of digestibility given for protein are apparent digestibility values inasmuch as no corrections were made for protein excretion of the mink. Calculations for protein digestibility coefficients were made as follows:

$$\%\ \text{indigestible protein} = \frac{(\text{Gm. feces})\,(\%\ \text{fecal protein})}{\text{Gm. protein consumed}}$$

$$\text{Coefficient of apparent digestibility} = 100\% - \%\ \text{indigestible protein}$$

Statistical analyses (analysis of variance) of the experimental data were conducted as described by Snedecor.[10]

RESULTS AND DISCUSSION

It is apparent from the experimental data presented (table 3), that fats are efficiently utilized by the mink. Although significant differences exist in the digestibility of the fats studied, these differences are not of practical importance relative to commercial ranch diets. The coefficients of digestibility of lard, tallow, fish fat, chicken

fat, and horse meat fat are almost identical to those found for the human species by Langworthy.[8] The digestibility values for horse meat fat and beef rumen fat are similar to those obtained by Inman *et al.*[6,7] for the fox.

TABLE 3—The Digestibility of Animal Fats by Mink

Fat source	Coefficient of true digestibility*
Processed fats	
Fish oil**	97.2 ± 0.4
Lard†	95.8 ± 0.5
Tallow‡	91.9 ± 0.6
Meat product fats	
Horse meat	95.5 ± 0.4
Chicken by-product	95.4 ± 0.4
Beef lung	93.7 ± 0.8
Beef rumen	93.2 ± 0.4
LSD§—0.05	1.5
0.01	2.0

* Mean and standard error of the mean. **Stabilized fish oil, VioBin Corp., Monticello, Ill. † Choice white grease, titer 37.2 C., and ‡ bleachable fancy tallow, titer 42.4 C., Oscar Mayer and Co., Madison, Wis. § Least significant difference between groups at 0.05 and 0.01 probability levels.

The data on the digestibility of proteins indicate that mink are not able to digest protein as efficiently as they digest fats. There is a considerable variation in the coefficients of digestibility of the proteins present in different animal by-products. An examination of the data on the digestibility of chicken by-products indicates that there may be a correlation between the ash or bone content of the protein source and the digestibility of the protein for the mink. Allison and Anderson,[2] in their study on the digestibility of flounder entrails and heads by the dog, observed a similar correlation between protein digestibility and ash content. The coefficient of digestibility for the protein of chicken feet is somewhat higher than the 45 per cent value obtained by Udall and McCay [11] on the digestibility of beef bone by the dog. The coefficients of digestibility for mink of the protein present in horse meat and beef rumen are lower than that obtained by Inman *et al.*[6,7] for the fox. The digestibility values for ocean whiting and beef rumen are similar to those obtained by Ahman in his studies with the mink.[1]

It is seen that both horse meat and chicken entrails are highly digestible for mink. The fact is of economic importance to the mink industry inasmuch as chicken by-products are obtainable at a far lower cost to the rancher than boneless horse meat.

A few comments are warranted on the palatability for the mink of the feed products studied. In general, all the products were readily accepted with the exception of the chicken feet. Only half of 20 mink offered the chicken feet diet would accept it. This complete refusal to consider the chicken feet diet as a source of nutrients was present even after a period of six days without alternative feed sources. Feed consumption of the animals that would accept the chicken feet diet was comparable to that of mink given the other digestion trial diets. In marked contrast to the poor palatability of the chicken feet was the high palatability of the chicken entrails for the mink. The observations on the digestibility and palatability of the various chicken by-products may be of value in practical mink nutrition. A highly digestible and palatable diet is desired for the optimum growth of young mink kits. Thus it may be advantageous for the rancher to include chicken entrails but not chicken feet in the diet used for the early growth phase of mink. The animal's requirements for calcium and phosphorus could be provided by the use of fish, chicken heads, or dicalcium phosphate in the diet.

TABLE 4—The Digestibility of Animal Proteins by Mink

Protein source	Coefficient of apparent digestibility*
Boneless horse meat	92.1 ± 0.2
Chicken entrails	89.3 ± 0.3
Ocean whiting	87.2 ± 0.2
Beef rumen	86.4 ± 0.7
Beef lungs	81.7 ± 0.5
Chicken heads	78.2 ± 0.6
Chicken feet	52.4 ± 1.1
LSD† — 0.05	1.7
0.01	2.3

* Mean and standard error of the mean. † Least significant difference between groups at 0.05 and 0.01 probability levels.

SUMMARY

1) Fish and animals fats are efficiently utilized by mink. Coefficients of digestibility for the fats tested were: fish oil, 97.2; lard, 95.8; tallow, 91.9; horse meat fat, 95.5; chicken fat, 95.4; beef lung fat, 93.7; and beef rumen fat, 93.2.

2) Protein digestibility coefficients for the products studied were: horse meat, 92.1; chicken entrails, 89.3; ocean whiting, 87.2;

beef rumen, 86.4; beef lungs, 81.7; chicken heads, 78.2; and chicken feet, 52.4.

3) Chicken entrails are highly palatable to mink while chicken feet have low palatability value.

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XUM

Scientific Notes

Serologic Classification of *Escherichia coli*

PAUL J. GLANTZ, Ph.D.

University Park, Pennsylvania

Escherichia coli has been an enigma to research workers for over a century. Attention has often been focused on the possible pathogenicity of *Esch. coli* strains. There has been considerable disagreement in regard to the methods by which pathogens are separated from nonpathogens. Morphological, cultural, and biochemical characteristics have proved to be inadequate. Early investigators despaired at the inconsistent results obtained with serologic methods.

When the characteristics of the *Esch. coli* K antigens were clarified, some of the causes of these difficulties were explained. This led to the establishment of an antigenic schema in which *Esch. coli* strains were classified according to their O (somatic), K (envelope or capsule), and H. (flagellar) antigens.

At present, the number and varieties of recognized *Esch. coli* antigens are as follows: O antigens, 139; K antigens, 84; and H antigens, 60. The O antigens are somatic and are not inactivated by heat at 100 C. or 121 C. The K antigens are somatic; occur as envelopes, sheaths, or capsules; and are divided into three varieties (L, A, B), based on their physical properties and behavior. While K antigens act as masking antigens, inhibiting agglutination of living suspensions in O antiserums, their inhibitory effects are inactivated by heat at 100 C. or 121 C. The H (flagellar) antigens are inactivated by heat at 100 C.

Since it is possible to subdivide many of the *Esch. coli* O antigens into two or more components, it is necessary to employ the terms O antigen group or O group. When the O, K, and H antigens of an *Esch. coli* strain are known, the term serotype is used; *i.e.*, serotype 026:B6:11. For accurate epidemiologic studies, complete serotyping is necessary. In this manner, identical serotypes are often reported from the same disease by different investigators. In some cases, unexplained "breaks" in isolation facilities may be due to the introduction of new *Esch. coli* strains, or *Esch. coli* strains having the same O antigens but different K or H antigens. When typed, these strains may be traced through the infected host to their source.

Escherichia coli serotypes have been isolated from calf scours, lamb dysentery, swine edema, mastitis, and some poultry diseases. Experimental transmission of some of these diseases has proved the pathogenicity assigned to certain *Esch. coli* strains. The same serotypes have been reported from calf scours by investigators in England, Germany, Denmark, India, Sweden, and the United States. Identical *Esch. coli* types were found in cases of lamb dysentery in Australia and England. The precise cause of swine edema remained unknown until similar *Esch. coli* types were isolated in Ireland, England, and the United States. Although mastitis and poultry diseases have not been studied extensively in regard to the *Esch. coli* types present, preliminary work indicates that certain *Esch. coli* types may be of importance.

From the many publications relating to the incidence of *Esch. coli* serotypes in infectious diseases of man and animals, it is now evident that not all members of the genus Escherichia should be considered as normal inhabitants of the intestinal tract. Much remains to be done in bacteriologic and serologic studies of *Esch. coli* serotypes. Unfortunately, the lack of facilities to provide complete serotyping is a handicap. There are only two such laboratories in the entire world; one in Denmark and one in the United States. It would be of tremendous value to those engaged in research if there was another serotyping laboratory in this country devoted primarily to *Esch. coli* types isolated from animal diseases.

Authorized for publication on June 30, 1959 as paper No. 2381 in the Journal Series of the Pennsylvania Agricultural Experiment Station, University Park.

Flocculation Tests for Pullorum Disease. II. A Study of the Stability of Frozen Antigen Extract for Conducting the Microscopic Slide Test

EDWARD P. ROZNOWSKI, Jr., M.S.
and V. D. FOLTZ, M.S.

Manhattan, Kansas

A study was made to determine the stability, in the frozen state, of an antigen extract previously described and reported.*

An aliquot of the material used in the previously reported work was frozen and stored at −18 C. for one year. Before study, the frozen extract was thawed at room temperature and tempered in a 37 C. water bath for 15 minutes. The antigen emulsion was prepared and the microscopic slide test was performed as in the original study.*

Contribution No. 350, from the Department of Bacteriology, Kansas Agricultural Experiment Station, Manhattan. Mr. Roznowski is now with Winthrop Laboratories, Myerstown, Pa.

* Roznowski, E. P., and Foltz, V. D.: Flocculation Tests for Pullorum Disease. Am. J. Vet. Res., *19*, (April, 1958): 478–482.

Known negative and positive antiserums with varying titers were used to compare the frozen, thawed, and tempered antigen extract with freshly prepared antigen extract.

The sensitivity and specificity of the frozen antigen extract was not impaired when used with positive serums having titers of 1:12.5 to 1:800.

Titers obtained with positive antiserums using antigen emulsions prepared from both the fresh and frozen antigen extract were identical.

There was no false positive reaction obtained with the antigen emulsion prepared from the frozen antigen extract.

Freezing appears to be a simple, inexpensive, practical method to preserve this particular antigen extract for at least one year.

International Association of Veterinary Food Hygienists

The Second Symposium of the International Association of Veterinary Food Hygienists will be held in Basel, Switzerland, May 15–21, 1960. The program will include lectures and discussions concerning minimum requirements for antemortem and postmortem inspection with reference to international acceptance, ionizing radiation in the preservation of foods and the disposition of meat that may contain radioactive material, bacteriologic examination and disposition of canned meat and fish products (both sterile and perishable), prepackaging, "public" slaughterhouses, milk hygiene in tropical and subtropical countries, milk sterilization by ultrahigh-temperature ash heating, and epidemiology of salmonellosis.

Contributions should be related to these subjects or other aspects of veterinary food hygiene; papers must be short (600 words or less) and written in one of the official languages (English, French, or German). The announcement deadline for contributions is Dec. 15, 1959.

Invitations, preliminary programs, application forms, and additional information may be obtained from Dr. C. H. Pals, vice-president, I.A.V.F.H., ARS, U.S.D.A., Washington 25, D.C.

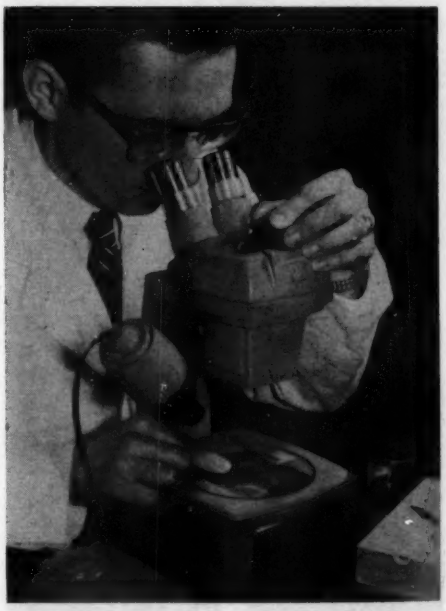
Research Tools

Further information on these products may be obtained by writing to: Research Tools, American Veterinary Medical Association, 600 S. Michigan Ave., Chicago 5, Ill.

New Stereomicroscope

A new stereomicroscope, manufactured by Bausch & Lomb Optical Co., allows continuous magnification through all stages without any image blackout when changing power.

The optical system is a single unit, easily reversible, which is completely sealed against dust and foreign materials. Four of these units, two of variable magnification and two of fixed power, give a magnification range of x 3.5 to x 120. They accommodate wide-field eyepieces of x 10, 15, or 20 and attachment lenses of x 0.5 and x 2.0. The working distance is constant at 4 inches without the use of lens attachments.

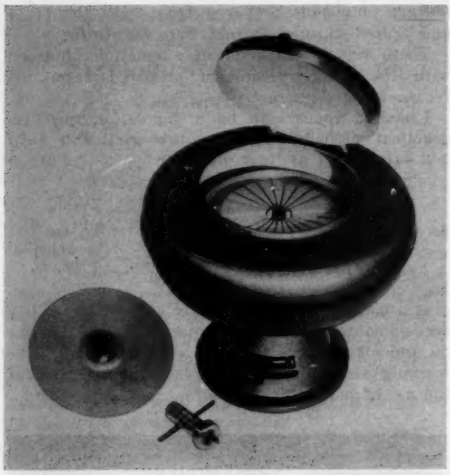


A special model, equipped with a dial micrometer, gives depths measurements that are precise to ± 0.1 mm. without touching the viewed object.

Microhematocrit Adapter

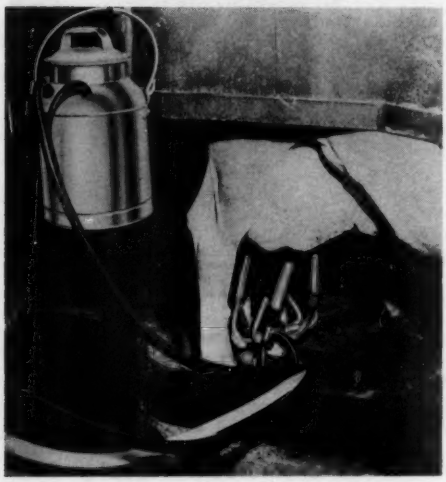
International clinical centrifuges can be easily adapted to spin microhematocrits with a Hemato-Kit manufactured by Arthur S. LaPine and Co.

The kit includes a 24-place head, with cover, for holding 24 capillary tubes, 1.5-mm. diameter by 75-mm. length. An adapter hub and lock for



attaching the head to the tapered shaft of a clinical centrifuge is also included. An adapted centrifuge will spin microhematocrits with complete packing of blood cells in 10 minutes at 7,500 r.p.m.

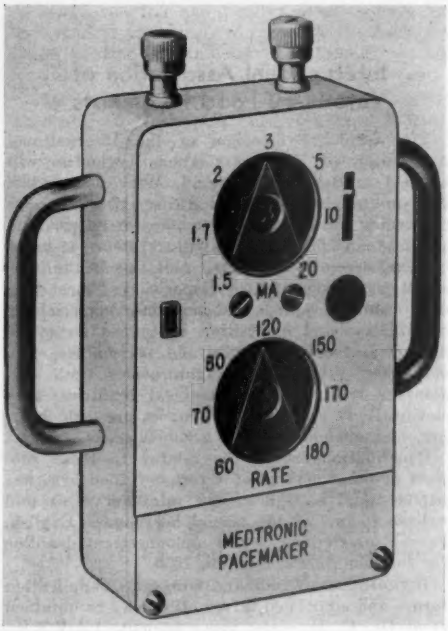
Quarter Milker



A device for milking infected quarters has been developed by the Premium Dairy Engineering Co. It consists of an 8-qt. trap with a self-locking cover and handle, a 4-ft. section of duplex rubber milk hose, and a team couple. It is adjustable to any teat cup or automatic milking machine.

Portable Transistor Cardiac Pacemaker

The Medtronic Cardiac Pacemaker, manufactured by Medtronic, Inc., is a compact (4 x 2½ x 1 in.) portable unit for the stimulation of ventricular function in cases of atrioventricular dissociation. It is designed for internal applications with at least one wire attached directly to the myocardium for temporary stimulation or with a bipolar patch for prolonged stimulation.



This 10-oz. instrument has a transistorized circuit; the power source has a 1,000-hour life and can be stored for a year or more without deterioration. Control knobs are recessed to prevent accidental change of the setting. The rate is calibrated in pulses per minute (60 to 180). The output control is calibrated in milliamperes, 1 to 20 ma. into a 350-ohm load, and the output pulse is a square wave of approximately 2 msec. duration. The Pacemaker will not interfere with the use of an electrocardiograph machine or other instruments that may be used simultaneously.

Prepared Plastic Plates

Plastic plates, prepared with a culture medium of mycosel agar, are for the diagnosis of fungus conditions and are available from the Fungus Diagnostic Services and Laboratory. The plates are ready for use, easily stored, and disposable.

The culture medium contains three antibiotics which selectively favor the growth of pathogenic fungi and retard and prevent the growth of bacteria and nonpathogenic fungi.

Disposable Plastic Caps and Hoods

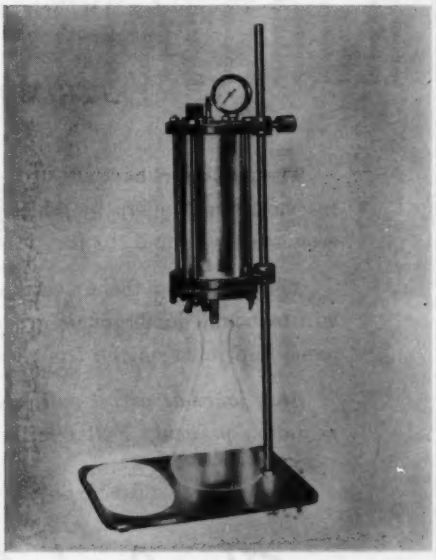
New inexpensive disposable caps and hoods of soft plastic are being offered by B. H. Jordan Co.

The cap can be worn as an insulation under a rigid helmet to prevent loose hair dust and dandruff contamination, or as a disposable shower cap for use in industries where contamination requires employee showering.

The hoods are designed to be worn as protection for the head and face against hazards which do not involve heavy impact and can, in some instances, be worn in place of goggles. They are made of acid-resistant, water- and weatherproof polyethylene.

Cylinder-Type Filter

Cylinder-type laboratory filters, manufactured by Ertel Engineering Corp., are available in stainless steel, hard rubber, plastic, or brass nickel-plated material.



A Pyrex glass cylinder permits full view of the product during the filtering process; it can be refilled without dissembling the unit. The filter will accommodate any type of filter medium, and the unit can be raised or lowered for vessels of various heights. A brass-plated pressure gauge for 0 to 60 p.s.i. is included as standard equipment.

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INDEX

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XUM

Index of Authors

Abare, Jean M., 966.
Affonso, Ottilia R., 247.
Alberts, J. O., 352.
Alexander, A. F., 680.
Alexander, G. I., 242.
Allen, Paul H., 278, 409, 442.
Allen, Robert C., 162.
Anderson, W. A., 324.
Appleton, George S., 166, 170.
Aronson, Arthur L., 909.

Bailey, J. W.,973.
Baker, Norman F., 201, 278, 409, 442.
Bankowski, R. A.,935, 941.
Barron, C. N., 1032.
Bay, R. C., 665.
Beal, G. A., 634, 872.
Beamer, Paul D., 858.
Beasley, J. N., 341.
Bell, J. Thomas, Jr., 53.
Bell, R. R., 766, 784.
Benson, Donald C., 917.
Bhannasiri, T., 756.
Bharadwaj, Madan B., 841.
Biberstein, Ernst L., 94.
Birkeland, J. M., 127, 568.
Bjornson, C. B., 985.
Black, Arthur L., 44.
Bogart, Ralph, 242,756.
Bohl, E. H., 127, 568, 592.
Boley, Loyd E., 858.
Booth, Stella, 1026.
Boucher, W. B., 287.
Bourke, J. M., 977.
Bowman, George W., 281, 487.
Brandly, C. A., 111, 148, 154, 946
Bratzler, J. W., 690.
Brisson, G. J., 338.
Brock, W. E., 297.
Brown, Albert L., 985.
Brown, J. M., 255.
Brown, R. R., 597.
Brudnjak, Z., 448.
Bustad, L. K., 14.

Calhoun, M. Lois, 61, 841.
Callis, J. J., 510.
Cameron, J. A., 350.
Campbell, L. A., 430.
Campeti, Frank L., 18.
Cartan, Gloria H., 235.
Carter, G. R., 173.
Casorso, D. Roy, 547.
Chang, P. W., 545.
Chaudhry, A. P., 1032.
Cheatham, W. J., 505.
Christensen, George C., 18.
Claflin, R. M., 634, 872.
Clark, Carl H., 1062, 1067, 1069.
Clark, David T., 452.
Clark, G. W., 708.
Clark, J. J., 1032.
Cohen, Daniel, 1026.
Coles, E. H., 832, 835, 838, 1020.
Connor, Nolen D., 452.
Corbo, L. J., 876.
Cornelius, Charles E., 44, 863, 917.
Couch, J. R., 961.
Cox, C. D., 747.
Crandell, R. A., 505.
Crook, Edward, 378.
Cunha, Raymundo G., 133, 138.
Cunningham, C. H., 876.

Dacres, W. G., 760.
Dale, C. N., 304, 311.
Dang, Hung Chen, 75.
Dardiri, A. H., 545.
Davis, Adamadia D., 27.
Davis, D. E., 341.
Davis, Leonard Reid, 281, 487.
Davis, Olive S., 119.
DeMott, Thomas, 287.
Dickson, W. M., 589.
DiLiello, L. R., 532.
Dimopoullos, George T., 270, 510.
Dixon, J. R., 884.
Djafar, M. I., 777.
Donker-Voet, Jeannette, 176.
Douglas, James R., 201, 278, 409, 442.
Drudge, J. H., 69, 670, 787.
Dunlap, J. S., 589.
Dunne, H. W., 615, 619, 690.
Durand, D., 116.

Easterday, Bernard C., 819.
Edward, A. G., 510, 992.
Eichhorn, Ervin A., 133.
Eisenstark, A., 116. 832. 835, 838.
Elam, G. W., 69, 670, 787.
Ellinghausen, H. C., Jr., 1072.
Epstein, Bernardo, 459.
Ersoy, Efem, 180.
Espana, Carlos, 795.
Espana, Ethel M., 795.
Evans, H. E., 702.
Eveland, Warren C., 1077.
Eveleth, D. F., 714.

Faber, J. E., 532.
Faustini, R., 483.
Fay, L. D., 925.
Fellowes, O. N., 510, 992.
Ferguson, L. C., 87.
Ferguson, T. M., 961.
Fernandes, Mario V., 398.
Ferrini, R., 483.
Fizette, W. B., 592.
Flint, Jean C., 33.
Flynn, Robert J., 198.
Foltz, V. D., 1090.
Foote, Lon E., 270.
Forsek, Z., 558.
Foster, N. M., 665
Frank, F. W., 973.
Frank, N. A., 718, 736.
Frederick, G. L., 333, 338.
Fritz, J. C., 655.
Fry, D. E., 545.

Galligan, Sylvia J., 430.
Galvin, T. J., 766, 784.
Gans, Joseph H., 909.
Getty, R., 573, 578.
Gill, Eugene, 357.
Gilmore, L. O., 736.
Gilroy, J. J., 677.
Ginevri, A., 625.
Glantz, P. J., 1090.
Goetsch, D. D., 697.
Goldberg, Aaron, 806.
Goldsberry, Steve, 61.
Gonzalez, Dolores, 795.
Gorczyca, Leonard R., 921.
Gorlin, R. J., 1032.
Gorman, George W., 192.
Grace, Oliver D., 239.
Graves, J. H., 510.
Gregory, P. W., 702.
Grimes, Roger D., 192.
Grumbles, L. C., 341.
Gustafson, Donald P., 119.

Habel, Robert E., 437.
Hadek, R., 573, 578, 753.
Haiba, M. H., 954.
Hamdy, A. H., 78, 84, 87.
Hammond, D. M., 708.
Hancock, B. B., 127.
Hansen, M. F., 662.
Hanson, L. E., 352, 537.
Hanson, R. P., 111, 819, 946.
Hawk, H. W., 206.
Hein, R. E., 662.
Herlich, Harry, 281, 487.
Hix, Elliott L., 184.
Ho, Pauline, 585.
Hoag, Warren G., 162.
Hokanson, J. F., 615.
Hollander, W. F., 750.
Hollister, C. J., 287.
Holtman, D. Frank, 350.
Hooper, L. L., 430.
Huebner, R. A., 287.
Hughes, J. S., 184.
Hundemann, A. S., 357.
Hutchings, L. M., 634.
Hutzler, L. B., 69, 670, 787.
Huygelen, C., 145.

Jarrett, W. F. H., 522, 527.
Jeffries, Charles D., 350.
Jennings, F. W., 522, 527.
Jensen, Elroy C., 898.
Jensen, Rue, 33, 677, 680.
Johnson, A. E., 708.
Johnson, V. L., 589.
Jones, L. D., 394.
Julian, L. M., 702.
Jungherr, Erwin L., 547.

Kaneko, Jiro J., 917.
Kantor, Sidney, 300.
Keeler, Richard F., 249.
Kennedy, Peter C., 94.
Kiesel, G. K., 760.
Kingrey, B. W., 255, 273.
Kitchell, R. L., 627.
Kleiber, Max, 44.
Kliewer, I. O., 297.
Krise, George M., 1081.
Kronfeld, D. S., 430.
Krueger, H. M., 242, 756.
Kuttler, Kenneth L., 434.

Lank, R.B., 273.
Leland, S. E., Jr., 69, 670, 787.
Leoschke, W. L., 1086.
Levine,Norman D., 300.
Lewis, R. E., 872.
Limon, Joe M., 921.
Lobl, K., 989.
Longhurst, William M., 201, 409.
Lozano, E. A., 394.
Lucas, Alfred M., 887.
Luedke, A. J., 615, 619, 690.

Maddy, K. T., 642.
Malhotra, Om P., 858.
Manning, John P., 858.
Manning, Robert, 242.
Marble, Donald W., 434.
Marquardt, W. C., 742.
Marshall, John D., 1077.
Matsuoka, Tats, 91.
Maurer, F. D., 505.
Mayhew, Roy L., 492, 498.
Mazzaracchio, V., 625.
McCarty, Robert T., 921.
McCrory, B. R., 665.
McCullough, N. B., 634, 872.
McDonald, L. E., 697.
McFarland, L. S., 702.
McFarland, R. H., 662.
McGowan, Blaine, Jr., 863.
McIntyre, W. I. M., 522, 527.
McKeever, Sturgis, 192.
McNutt, S. H., 378.
Meinershagen, W. A., 973.
Melendez, Luis V., 815.
Miller, Grover C., 492.
Miller, V. A., 677.
Miner, M. L., 708.
Mitidieri, Emilio, 247.
Moll, Torbjorn, 27.
Moore, R. W., 884.
Morgan, C. L., 639.
Morgan, H. C., 372.
Mortelmans, J., 145.
Moscovici, C., 625.
Mosier, J. E., 1020.
Moulton, Jack E., 863.
Mulligan, W., 522. 527.
Munday, B. L., 977.
Muth, O. H., 231.

Nichols. R. E., 445.
Njoku-Obi, A., 966.
Norton, Horace W., 180, 585, 858.

Obel, Anna-Lisa, 384.
Odell, G., 697.
O'Halloran, M. W., 977.
Oldfield, J. E., 231.
Osebold, John W., 966.

Page, L. A., 935, 941.
Pamukcu, A. M., 597.
Parker, W. D., 394.
Pearson, C. C., 297.
Penn, Katherine, 445.
Pensinger, Robert R., 180.

Park, K., 989.
Poelma, L. J., 532.
Poppensiek, G. C., 510, 992.
Pounden, W. D., 78, 87, 718, 736.
Price, J. M., 597.

Reber, Elwood F., 180, 585, 858.
Reid, Winifred B., 825.
Remmert, L. F., 231.
Rhoades, H. E., 537.
Richey, D. J., 659.
Richter, Ward R., 603.
Rigdon, R. H., 647, 961.
Ristic, Miodrag, 148, 154.
Roepke, Martin H., 33.
Rogols, S., 592.
Romic, Z., 558.
Rosenquist, B. D., 255.
Rosenwald, A. S., 946.
Roznowski, Edward P., Jr.,1090.

Sanger, V. L., 84, 718.
Savan, M., 996.
Schipper, I. A., 714.
Schoene, R. B., 655.
Schrader, Gene T., 270.
Schubert, J. R., 231.
Schubert, Joseph H., 192.
Schwartz, Benjamin, 7.
Scrivner, L. H., 973.
Seigneur, L. J., 14.
Selim, M. K., 954.
Sellers, A. F., 461.
Senseman,V F., 777.
Serrao, Ubiratan M., 138.
Sertic, V., 448.
Sharp, J. B., 992.
Sharp, N. C. C., 522, 527.
Shuman, R. D., 1002.
Simon, Joseph, 819.
Singh, K. V., 568.
Skerman, K. D., 977.
Smidt, M. J., 655.
Smith, Chauncey W., 1077.
Smith, Louis DS, 91.
Songer, J. R., 304, 311.
Staley, E. E., 297.
Stevens, C. E., 461.
Stone, H. D., 357.
Stromberg, M. W., 319, 627.
Sullivan, D. J., 324.
Sullivan, J. F., 357.
Sussman, Oscar, 1026.
Sutherland, A. K., 977.
Swanson, L. E., 777.
Swingle, Karl F., 75, 235, 249.
Switzer, William P., 1010.

Talanti, S., 41.
Taylor, C. B., 634.
Taylor, Gale D., 300.
Terhaar, C. J., 662.
Tessler, J., 992.
Test, L. D., 14.
Tokarnia, Carlos Hubinger, 247.
Torbert, B. J., 492.
Torturella, Ivo, 138.
Trost, W. A., 708.
Trotter, D. M., 213.
Turk, R. D., 766, 784.
Turner, James H., 102.
Twiehaus, M. J., 562.
Tyler, W. S., 702.
Udall, R. H., 423, 426.
Underbjerg, G. K. L., 184.
Underdahl, Norman, 239.
Urquhart, G. M., 522.

Vaghi, M. A., 483.
Vatter, A. E., 537, 723.

Wade, A. E., 777.
Wald, Niel, 1081.
Watkins, J. R., 884.
Watrach, A. M., 537, 723.
Watrach, Marian A., 537.
Wayt, L. Keith, 265.
Weaver, C. R., 736.
Weber, Alvin F., 53.
Weide, Kenneth D., 562.
Wharton, F. D., 655.
White, Philip G., 166, 170.
Wilcox, F. H., 957.
Wilson, J. B., 825.
Winter, Hans, 366.
Woodley, C. H., 1062, 1067, 1069.
Wyant, Z. N., 69, 670, 787.

Yates, V. J., 545.
Youatt, W. G., 925.
Young, George A., 239.
Young, Stuart, 75.
Yusken, John W., 585.

Zeljko, M., 558.
Zeskov, B., 448, 852.
Zuschek, F., 111.

Index of Subjects

A

Abscesses in the Horse, Radiographic Diagnosis of Mesenteric, 448.
Acariasis of Mice Caused by Psorergates-Simplex Successfully Treated with Aramite, Follicular, 198.
Achondroplastic Dwarfism in Cattle, Two Projections onto the Cranial Cavity Associated with, 702.
Acidosis and Plasma Glucose in Sheep Given Butyrate, 430.
Adrenal Glands of Mature Nonpregnant Dairy Heifers, Nonpregnant Lactating Dairy Cows, and Pregnant Lactating Dairy Cows, A Comparative Study of Lipid Accumulation in the, 53.
Analgesics and Their Antagonists as Adjuncts to Barbiturate Anesthesia, The Narcotic. I. Physiological and Pharmacological Considerations, 909.
Anaplasma Marginale. I. Studies with Phase Contrast and Electron Microscopy, 795.
Anaplasmosis, The Screening of Five Drugs for Their Activity Against Bovine, 297.
Anemia, Feline Infectious. II. Experimental Cases, 33.
Anesthesia, The Narcotic Analgesics and Their Antagonists as Adjuncts to Barbiturate. I. Physiological and Pharmacological Considerations, 909.
Angora Goat, Observations on the Penile Development of the, 603.
Antelope, Using Glycine-2-C^{14}, Erythrocyte Survival Studies in the Mule Deer, Aoudad Sheep, and Springbok, 917.
Anthelmintic Efficiency of Purified and N.F. Phenothiazine, A Comparison of the, 409.
Anthelmintic in Cattle, Trial with a New Organic Phosphate as an, 278.
Anthelmintic in Sheep, Trial with a New Organic Phosphate as an, 442.
Anthelmintic Studies with Carbon 14-Labeled Carbon Tetrachloride on Ascaridia Galli (Nematoda) and Its Chicken Host, 662.
Anthelmintics in Ruminants, The Efficacy and Toxicity of Certain Organic Phosphates and a Carbamide as, 784.
Antibiotics for the Control of Vibriosis of Ewes, 973.
Antibiotics on Semen Production in Rabbits, The Effect of, 756.
Antibiotics, Symposium on (Scientific Note), 766, 927.
Antigenicity Studies of Heated Tissue Suspensions Containing Foot-and-Mouth Disease Virus, Thermal Inactivation and, 510.
Appetite in Adult Ewes, The Effect of Intravenous Glucose Injections on, 242.
Aramite, Follicular Acariasis of Mice Caused by Psorergates-Simplex Successfully Treated with, 198.
Artery in Calves, Experimental Stenosis of the Pulmonary, 265.
Ascarid-Infected, and Hog Cholera-Vaccinated Swine, Hematological Studies of Normal, 562.
Ascaridia Galli (Nematoda) and Its Chicken Host, Anthelmintic Studies with Carbon 14-Labeled Carbon Tetrachloride on, 662.
Ascaris Suum, Experimental Infection of Pigs with, 7.
Avian Infectious Laryngotracheitis, Electron Microscopic Studies of the Virus of, 537.
Avian Lymphomatosis, Conference on Histopathology of Experimental (Scientific Note), 223.
Avian Visceral Lymphoid Tumors and in Vitro Serial Passage of the Virus, Tissue Culture of, 119.

B

Bacteremia in Pregnant Sheep Following Oral Administration of Vibrio Fetus, 677.
Barbiturate Anesthesia, The Narcotic Analgesics and Their Antagonists as Adjuncts to. I. Physiological and Pharmacological Considerations, 909.
Benzathine Penicillin V in Bovine Mastitis, Parenteral, 287.
Beta-Propiolactone, The Inactivation of Foot-and-Mouth Disease Virus, Type A-119, with, 992.
(Bloat) Frothing of Samples of Ruminal Digesta Following the Addition of Certain Nonvolatile Organic Acids of Legumes, Increases in the, 445.
Blood Cells After Parenteral Injection at Various Sites, The Absorption of Red, 1062.
Blood Cells, The Effects of Certain Diseases and Transfusion Methods on the Life Span of Red, 1069.
Blood Glucose and Plasma Sodium and Potassium in the Cow, The Effects of Four Synthetic Corticosteroids on Leukocytes, 697.
Blood Loss in the Macaca Mulatta Monkey, Hematological Effects of Acute and Chronic Experimental, 1081.
Blood Serum Levels in Swine, The Effect of Intramuscular Injections of Vitamin B_{12} on, 333.
Blood Serum Vitamin B_{12} Levels in Swine, Influence of Oral Intake of Vitamin B_{12} on Total, 338.
Blood Volumes as Measured by Rose Bengal, T-1824 (Evans Blue), Radiochromium-Tagged Erythrocytes, and

XUM

a Combination of the Latter Two, A Comparison of, 1067.
Bluetongue Virus in Tissue Culture, Isolation and Propagation of, 398.
Bluetongue Virus, Virucidal Effect of Some Chemical Agents on, 665.
Body Fluids of Ruminants and Their Simultaneous Determination, The, 184.
Botulism, Laboratory Evaluation of a Trivalent Toxoid for, 170.
Bovine Coccidiosis with Multiple Small Doses and Single Large Doses of Sulfamethazine and Sulfabromomethazine, Treatment of Experimental, 708.
Bovine Gastrointestinal Parasites, Studies on. XX. The Results of Feeding Small Amounts of Phenothiazine in Pure Infections of Cooperia Punctata, 492.
Bovine Gastrointestinal Parasites, Studies on. XXII. Rate of Consumption of Phenothiazine in Salt-Phenothiazine Mixtures by Cattle, 498.
Bovine Leptospirosis, Evaluation of the Hemolytic Test in Canine and, 747.
Bovine Male, Histological Observations of the Genitalia of the Immature, the Castrated, and the Mature, 213.
Bovine Mastitis, Experimental Viral, 819.
Bovine Rhinotracheitis—The Occurrence of Intranuclear Inclusion Bodies in Experimentally Infected Animals, Infectious, 505.
Bovine Rhinotracheitis Virus, The Relationship of Nasal Discharge to Infection with Infectious, 985.
Bovine Serum, Electrophoretic Studies of. I. Changes in the Serum Proteins After Splenectomy, 270.
Bovine Udder, A Study of the Staphylococci Associated with the, 825.
Bovine Urine, Identification and Determination of Kynurenin and 3-Hydroxykynurenin in, 597.
(Brisket Disease) and in Experimental Cattle Maintained at High Altitudes, Gross Cardiac Changes in Cattle with High Mountain, 680.
Bromosulfamerazine and Bromosulfamethazine, The Pharmacological Action Developed in Cattle by Two Halogenated Sulfapyrimidines—, 483.
Bromosulfamethazine, The Pharmacological Action Developed in Cattle by Two Halogenated Sulfapyrimidines—Bromosulfamerazine and, 483.
Bronchitis Virus, Hemagglutination by Trypsin-Modified Infectious, 876.
Brucella Abortus, Studies on an Immunogenic Agent Against. I. Titrations of a New Soluble Immunogenic Agent Against Experimental Infections in Guinea Pigs, 162.
Brucella Melitensis (Mediterranean Type), Experimental Infection of Swine with, 634.
Brucella Neotomae, Experimental Infection of Swine with, 872.
Brucellosis in White-Tailed Deer, Experimental, 925.
Butyrate, Acidosis and Plasma Glucose in Sheep Given, 430.

C

Calculi of Bovine Origin, A Partial Chemical Analysis of the Mucoprotein of Siliceous Urinary, 249.
Calculosis, Preliminary Observations in Experimental Ovine. Ruminant Urolithiasis. I., 863.
Calves—Double Vaccination with Irradiated Larvae, Immunological Studies on Dictyocaulus Viviparus Infection in, 522.
Calves, Experimental Stenosis of the Pulmonary Artery in, 265.
Calves with Coccidia and Nematodes, Studies on Experimental Concurrent Infections of Dairy. I. Eimeria Spp. and the Small Intestinal Worm, Cooperia Punctata, 281.
Camera, Recording (Research Tool), 768.
Canine and Bovine Leptospirosis, Evaluation of the Hemolytic Test in, 747.
Canine Autogenous Skin Grafting, 898.
Canine Bacterial Nephritis, Effect of Nitrofurantoin on Experimental, 1020.
Canine Dirofilariasis, The Pathology of, 366.
Canine Liver Dysfunction, A Comparison of Uric Acid Determinations and Sulfobromophthalein Sodium Retention Tests as an Index to, 372.
Canine Lymphoma and Its Public Health Significance, An Epidemiological Study of, 1026.
Carbamates and Related Compounds Against Horse Strongyle Larvae in Vitro, Screening Tests of, 300.
Carbamide as Anthelmintics in Ruminants, The Efficacy and Toxicity of Certain Organic Phosphates and a, 784.
Carbon Tetrachloride on Ascaridia Galli (Nematoda) and Its Chicken Host, Anthelmintic Studies with Carbon 14-Labeled, 662.
Cardiac Changes in Cattle with High Mountain (Brisket Disease) and in Experimental Cattle Maintained at High Altitudes, Gross, 680.
Cataracts in Chickens with Lymphomatosis, 647.
Cataracts in Turkeys, Spontaneous, 961.
Cattle and Sheep from Northeastern and Northern Brazil, Copper and Iron in Livers of Undernourished, 247.
Cattle by Cobalt Pellet Therapy, The Correction of Cobalt or Vitamin B_{12} Deficiency in, 977.
Cattle in Alabama, A Study of Infertility in, 760.
Cattle in Texas, Incidence of Certain Gastrointestinal Parasites of, 766.
Cattle, Serum Cholesterol and Paper Electrophoretic Lipoprotein in, 989.
Cattle, Sodium Metabisulfite and Sulfur Dioxide Gas (Silage Preservative) Poisoning in, 690.
Cattle, The Comparative Histology of the Skin of Hereford and Aberdeen Angus, 61.
Cattle, The Morphology and Sporulation of the Oocyst of Eimeria Brasiliensis, Torres and Ramos 1939, of, 742.
Cattle, The Relationship of Diet to Gastrointestinal Helminth Parasitism in, 806.
Cattle, Two Projections onto the Cranial Cavity Associated with Achondroplastic Dwarfism in, 702.
Cattle with High Mountain (Brisket Disease) and in Experimental Cattle Maintained at High Altitudes, Gross Cardiac Changes in, 680.
Cattle with Respiratory Disease, Isolation and Characterization of Cytopathogenic Enteroviruses from, 27.
Chicken Embryo to Certain Avian Pathogens, The Response of the Developing, 547.
Chicken Host, Anthelmintic Studies with Carbon 14-Labeled Carbon Tetrachloride on Ascaridia Galli (Nematoda) and Its, 662.
Chicken Spermatozoa, Studies of the Effect of Oxytetracycline on, 957.
Chickens, Newcastle Disease Virus in the Aqueous Humor of, 545.
Chickens to Salmonella Pullorum, Susceptibility of Certain Strains of White Leghorn, 350.
Chickens with Lymphomatosis, Cataracts in, 647.
Chicks and Poults, The Effect of Para-Aminobenzoic Acid on the Toxicity of 3-Nitro-4-Hydroxyphenylarsonic Acid in, 655.
Chloramphenicol and Furazolidone, The Treatment of Salmonella Gallinarum Infection in Turkey Poults with, 659.
Chorioallantoic Membranes Suspended in Tyrode's Solution, Influence of Temperature on the Growth of Newcastle Disease Virus in, 111.
Clostridium Botulinum Type C Toxoids in Mink, Field Evaluation of, 166.
Clostridium Perfringens Using Nonactivated Vaccine, Maternally Induced Protection of Young Lambs Against the Epsilon Toxin of, 91.
Cobalt or Vitamin B_{12} Deficiency in Cattle by Cobalt Pellet Therapy, The Correction of, 977.
Coccidia and Nematodes, Studies on Experimental Concurrent Infections of Dairy Calves with. I. Eimeria Spp. and the Small Intestinal Worm, Cooperia Punctata, 281.
Coccidia and Nematodes, Studies on Experimental Concurrent Infections of Dairy Calves with. II. Eimeria Spp. and the Medium Stomach Worm, Ostertagia Ostertagi, 487.
Coccidioides Immitis Infection, A Study of a Site in Arizona Where a Dog Apparently Acquired, 642.
Coccidiosis with Multiple Small Doses and Single Large Doses of Sulfamethazine and Sulfabromomethazine, Treatment of Experimental Bovine, 708.
Complement-Fixation Test in the Study of Foot-and-Mouth Disease, An Evaluation of the, 996.
Container, Escape-Proof (Research Tool), 414.
Cooperia Punctata, Eimeria Spp. and the Small Intestinal Worm. Studies on Experimental Concurrent Infections of Dairy Calves with Coccidia and Nematodes. I., 281.
Cooperia Punctata, The Results of Feeding Small Amounts of Phenothiazine in Pure Infections of. Studies on Bovine Gastrointestinal Parasites. XX., 492.
Coordinate Plate and Film Comparator (Research Tool), 224.
Copper and Iron in Livers of Undernourished Cattle and Sheep from Northeastern and Northern Brazil, 247.
Coronary Circulation in the Dog and Pig, Anatomic and Functional Studies of the, 18.
Corticosteroids on Leukocytes, Blood Glucose, and Plasma Sodium and Potassium in the Cow, The Effects of Four Synthetic, 697.
Cow, Rates and Routes of Sulfonamide Excretion in the. I. Milk Levels Following Single Intravenous and Oral Administrations, 714.
Cow, The Effects of Four Synthetic Corticosteroids on Leukocytes, Blood Glucose and Plasma Sodium and Potassium in the, 697.

Cows, A Comparative Study of Lipid Accumulation in the Adrenal Glands of Mature Nonpregnant Dairy Heifers, Nonpregnant Lactating Dairy Cows, and Pregnant Lactating Dairy, 53.
Cows, Electrocardiograms of Normal, Lactating Dairy, 273.
Cows, The Use of Glycine-1-C^{14} in the Measurement of Serum Protein Turnover Rates in Dairy, 44.
Crematory Destructor, Medical (Research Tool), 223.
Cyanacethydrazide as a Treatment of Lungworm, Dictyocaulus Viviparus (Bloch), Infections in Cattle, The Efficacy of, 777.

D

Deer, Aoudad Sheep, and Springbok Antelope, Using Glycine-2-C^{14}, Erythrocyte Survival Studies on the Mule, 917.
Deer, Experimental Brucellosis in White-Tailed, 925.
Dictyocaulus Viviparus (Bloch), Infections in Cattle, The Efficacy of Cyanacethydrazide as a Treatment of Lungworm, 777.
Dictyocaulus Viviparus Infection in Calves—Double Vaccination with Irradiated Larvae, Immunological Studies on, 522.
Diet to Gastrointestinal Helminth Parasitism in Cattle, The Relationship of, 806.
Dirofilariasis, The Pathology of Canine, 366.
Distemper in Mink and Ferrets, Experimental. II. Appearance and Significance of Histopathological Changes, 378.
Distillation Unit, New (Research Tool), 415.
Dog and Pig, Anatomic and Functional Studies of the Coronary Circulation in the, 18.
Dog Apparently Acquired Coccidioides Immitis Infection, A Study of a Site in Arizona Where a, 642.
Dogs, with Reference to the Hypothalamic-Hypophysial Neurosecretory System, Observations on Pyometra in, 41.
Drugs for Their Activity Against Bovine Anaplasmosis, The Screening of Five, 297.
Dwarfism in Cattle, Two Projections onto the Cranial Cavity Associated with Achondroplastic, 702.

E

Egrets, Experimental Ornithosis in Herons and, 884.
Ehrlich Cells, Western Equine Encephalomyelitis Virus in Tissue Culture and Color Test Titration of Virus and Its Antibodies in, 459.
Eimeria Brasiliensis, Torres and Ramos 1939, of Cattle, The Morphology and Sporulation of the Oocyst of, 742.
Eimeria Maxima, A Study on the Oocystic Sporulation of Some Avian and Mammalian Eimeria Species Under Simulated Egyptian Summer Conditions, with Special Reference to the Cycle of Oocyst Production of, 954.
Eimeria Species Under Simulated Egyptian Summer Conditions, with Special Reference to the Cycle of Oocyst Production of Eimeria Maxima, A Study on the Oocystic Sporulation of Some Avian and Mammalian, 954.
Eimeria Spp. and the Medium Stomach Worm, Ostertagia Ostertagi, Studies on Experimental Concurrent Infections of Dairy Calves with Coccidia and Nematodes. II., 487.
Eimeria Spp. and the Small Intestinal Worm, Cooperia Punctata, Studies on Experimental Concurrent Infections of Dairy Calves with Coccidia and Nematodes. I., 281.
Eimeria Stiedae, Ionographic Studies of Rabbits Infected with, 589.
Electrocardiogram Marker, Transistor (Research Tool), 927.
Electrocardiograms of Normal, Lactating Dairy Cows, 273.
Electrode Assembly, Miniature pH (Research Tool), 416.
Electron Microscopy of the Pulmonary Alveolar Wall, 723.
Electrophoresis, The Effects of Freezing and Storage at —20 C. on Serum Protein Patterns of Sheep as Determined by Paper, 434.
Embryo to Certain Avian Pathogens, The Response of the Developing Chicken, 547.
Embryonal Nephroma in Swine, 324.
Encephalomyelitis Virus in Tissue Culture and Color Test Titration of Virus and Its Antibodies in Ehrlich Cells, Western Equine, 459.
Enteritis, Isolation of a Cytopathogenic Agent from Swine with, 625.
Enteroviruses from Cattle with Respiratory Disease, Isolation and Characterization of Cytopathogenic, 27.
Enteroviruses, The Use of the Plaque Technique for the Study of Porcine, 568.
Equine Encephalomyelitis Virus in Tissue Culture and Color Test Titration of Virus and Its Antibodies in Ehrlich Cells, Western, 459.
Eructation Reflex, Studies of the Reflex Control of the Ruminant Stomach with Special Reference to the, 461.
Erysipelas Bacterins and Vaccines in Weanling Pigs, with Particular Reference to the Status of Their Dams, Comparative Experimental Evaluation of Swine, 1002.
Erysipelas Serum-Culture Agglutination (ESCA) Test, An, 394.
Erysipelas with Adsorbed Bacterin, Interference of Anti-Hog Cholera Hyperimmune Serum with the Immunity in Mice Vaccinated Against Swine, 538.
Erysipelothrix Insidiosa with Fluorescent Antibody, The Identification of Viable and Nonviable, 1077.
Erythrocyte Survival Studies in the Mule Deer, Aoudad Sheep, and Springbok Antelope, Using Glycine-2-C^{14}, 917.
Erythrocytes, and a Combination of the Latter Two, A Comparison of Blood Volumes as Measured by Rose Bengal, T-1824 (Evans Blue), Radiochromium-Tagged, 1067.
Escherichia Coli, Serologic Classification of (Scientific Note), 1090.
Ewes, Antibiotics for the Control of Vibriosis of, 973.
Ewes, Phenolsulfonphthalein Clearance as a Measure of Renal Function in, 858.
Ewes, The Effect of Intravenous Glucose Injections on Appetite in Adult, 242.

F

Fats and Proteins by Mink, The Digestivity of Animal, 1086.
Feline Infectious Anemia. II. Experimental Cases, 33.
Ferrets, Experimental Distemper in Mink and. II. Appearance and Significance of Histopathological Changes, 378.
Fibula in the Horse, A Study of Discontinuity of the, 852.
Flame Photometer (Research Tool), 607.
Flocculation Tests for Pullorum Disease. II. A Study of the Stability of Frozen Antigen Extract for Conducting the Microscopic Slide Test (Scientific Note), 1090.
Flury Vaccine and Subsequent Challenge with Fixed Rabies Virus, Attempts to Immunize Guinea Pigs with, 145.
Foals for Gastrointestinal Parasites, Field Tests on the Efficacy of Piperazine-Carbon Disulfide Complex in the Treatment of, 452.
Foot-and-Mouth Disease, An Evaluation of the Complement-Fixation Test in the Study of, 996.
Foot-and-Mouth Disease Virus in Cultures of Fetal Rabbit Lung Cells, Multiplication and Cytopathogenic Effect on, 815.
Foot-and-Mouth Disease Virus, Studies on Rabbit-Adapted. I. Propagation and Pathogenicity, 133.
Foot-and-Mouth Disease Virus, Studies on Rabbit-Adapted. II. Immunogenicity, 138.
Foot-and-Mouth Disease Virus, Thermal Inactivation and Antigenicity Studies of Heated Tissue Suspensions Containing, 510.
Foot-and-Mouth Disease Virus, Type A-119, with Beta-Propiolactone, The Inactivation of, 992.
Freezing and Storage at —20 C. on Serum Protein Patterns of Sheep as Determined by Paper Electrophoresis, The Effects of, 434.
Frothing of Samples of Ruminal Digesta Following the Addition of Certain Nonvolatile Organic Acids of Legumes, Increases in the, 445.
Furazolidone, The Treatment of Salmonella Gallinarum Infection in Turkey Poults with Chloramphenicol and, 639.

G

Gastroenteritis Virus, The Effects of Infecting Newborn Pigs with Transmissible, 585.
Gastrointestinal Parasites, Field Tests on the Efficacy of Piperazine-Carbon Disulfide Complex in the Treatment of Foals for, 452.
Gastrointestinal Parasites of Cattle in Texas, Incidence of Certain, 766.
Gastrointestinal Parasites, Studies on Bovine. XX. The Results of Feeding Small Amounts of Phenothiazine in Pure Infections of Cooperia Punctata, 492.
Gastrointestinal Parasites, Studies on Bovine. XXII. Rate of Consumption of Phenothiazine in Salt-Phenothiazine Mixtures by Cattle, 498.
Genitalia of the Immature, the Castrated, and the Mature Bovine Male, Histological Observations of the, 213.
Glucose Injections on Appetite in Adult Ewes, The Effect of Intravenous, 242.
Glycine-1-C^{14} in the Measurement of Serum Protein Turnover Rates in Dairy Cows, The Use of, 44.
Glycine-2-C^{14}, Erythrocyte Survival Studies in the Mule Deer, Aoudad Sheep, and Springbok Antelope, Using, 917.

XUM

Goat Serum Proteins by Paper Electrophoresis, Studies of, 921.
Goats, Experimental Strongyloidiasis in Sheep and. I. Single Infections, 102.
Guinea Pigs, Titrations of a New Soluble Immunogenic Agent Against Experimental Infections in. Studies on an Immunogenic Agent Against Brucella Abortus. I., 162.
Guinea Pigs with Flury Vaccine and Subsequent Challenge with Fixed Rabies Virus, Attempts to Immunize, 145.

H

Haemonchus Contortus, Efficacy of Single Therapeutic Doses for the Removal of. Strain Variation on the Response of Sheep Nematodes to the Action of Phenothiazine. IV., 670.
Haemonchus Contortus Infection—Vaccination of Sheep Using a Single Dose of X-Irradiated Larvae, Studies on Immunity to, 527.
Helminth Parasitism in Cattle, The Relationship of Diet to Gastrointestinal, 806.
Hemagglutination by Trypsin-Modified Infectious Bronchitis Virus, 876.
Hematological Nomenclature, A Discussion of Synonymy in Avian and Mammalian, 887.
Hematological Studies of Normal, Ascarid-Infected, and Hog Cholera-Vaccinated Swine, 562.
Hematology of Chronic Bovine Reticuloperitonitis, The, 255.
Hemoglobinometer (Research Tool), 928.
Herons and Egrets, Experimental Ornithosis in, 884.
High Mountain (Brisket Disease) and in Experimental Cattle Maintained at High Altitudes, Gross Cardiac Changes in Cattle with, 680.
Hog Cholera Hyperimmune Serum with the Immunity in Mice Vaccinated Against Swine Erysipelas with Adsorbed Bacterin, Interference of Anti-, 558.
Hog Cholera, The Pathogenesis of. I. Route of Entrance of the Virus into the Animal Body, 615.
Hog Cholera, The Pathogenesis of. II. The Virus Eclipse Phase and Sensitization of the Host, 619.
Hog Cholera-Vaccinated Swine, Hematological Studies of Normal, Ascarid-Infected, and, 562.
Hog Cholera Virus, In Vitro Propagation of. II. Some Biological and Immunological Characteristics of Hog Cholera Virus Grown in Tissue Culture, 304.
Hog Cholera Virus, In Vitro Propagation of. III. Cultivation of an Immunological Variant, with Retention of Its Identifying Characteristics, 311.
Horse, A Study of Discontinuity of the Fibula in the, 852.
Horse, Critical Tests with Polymethylenepiperazine (PMP) Against Parasites of the, 69.
Horse, Radiographic Diagnosis of Mesenteric Abscesses in the, 448.
Horse Strongyle Larvae in Vitro, Screening Tests of Carbamates and Related Compounds Against, 300.
3-Hydroxykynurenin in Bovine Urine, Identification and Determination of Kynurenin and, 597.
Hysterectomy and Their Isolation, Procurement of Lambs by, 239.

I

Incubator, Bacteriological (Research Tool), 608.
Infertility in Cattle in Alabama, A Study of, 760.
Instrument Symposium and Research Equipment Exhibit (Scientific Note), 927.
Intussusception in the Mouse, Colonic, 750.
Iron in Livers of Undernourished Cattle and Sheep from Northeastern and Northern Brazil, Copper and, 247.
Irradiated Larvae, Immunological Studies on Dictyocaulus Viviparus Infection in Calves—Double Vaccination with, 522.
Irradiated Larvae, Studies on Immunity to Haemonchus Contortus Infection—Vaccination of Sheep Using a Single Dose of X-, 527.

K

Kidney Cell Cultures—Susceptibility to Viruses and Use in Isolation of Enteric Viruses of Swine, Swine, 127.
(Kidney) Phenolsulfonphthalein Clearance as a Measure of Renal Function in Ewes, 858.
Kynurenin and 3-Hydroxykynurenin in Bovine Urine, Identification and Determination of, 397.

L

Lamb Pneumonia, Characteristics of a Virus Associated with, 84.
Lambs Against the Epsilon Toxin of Clostridium Perfringens Using Nonactivated Vaccine, Maternally Induced Protection of Young, 91.
Lambs by Hysterectomy and Their Isolation, Procurement of, 239.
Lambs, Experimental Production of Pneumonia in, 78.
Lambs, Microbial Agents Associated with Pneumonia in Slaughtered, 87.
Lambs, Septicemic Pasteurellosis in, 94.
Lambs, The Relationship of Serum Glutamic Oxalacetic Transaminase to Nutritional Muscular Dystrophy in, 75.
Laryngotracheitis, Electron Microscopic Studies of the Virus of Avian Infectious, 537.
Legumes, Increases in the Frothing of Samples of Ruminal Digesta Following the Addition of Certain Nonvolatile Organic Acids of, 445.
Leptospira Pomona, Studies on the Relationship of a Phospholipase to the Hemolysin of, 592.
Leptospires, Nephelometry and a Nephelo-Culture Flask Used in Measuring Growth of, 1072.
Leptospirosis, Evaluation of the Hemolytic Test in Canine and Bovine, 747.
Leptospirosis in Wild Mammals, Comparison of Bacteriological and Serological Techniques for Detection of, 192.
Leukocytes, Blood Glucose and Plasma Sodium and Potassium in the Cow, The Effects of Four Synthetic Corticosteroids on, 697.
Lipid Accumulation in the Adrenal Glands of Mature Nonpregnant Dairy Heifers, Nonpregnant Lactating Dairy Cows, and Pregnant Lactating Dairy Cows, A Comparative Study of, 53.
Lipids in the Epithelium of the Ruminant Forestomach, The Presence of, 437. Correction: 783.
Lipoprotein in Cattle, Serum Cholesterol and Paper Electrophoretic, 989.
Listeria Monocytogenes and Pilot Studies on Vaccination, Acquired Resistance of Sheep to, 966.
Listeria Monocytogenes Isolated in Michigan, A Serological Study on Some Strains of, 176.
Liver Dysfunction, A Comparison of Uric Acid Determinations and Sulfobromophthalein Sodium Retention Tests as an Index to Canine, 372.
Livers of Undernourished Cattle and Sheep from Northeastern and Northern Brazil, Copper and Iron in, 247.
Lungworm, Dictyocaulus Viviparus (Bloch), Infections in Cattle, The Efficacy of Cyanacethydrazide as a Treatment of, 777.
Lymphoma and Its Public Health Significance, An Epidemiological Study of Canine, 1026.
Lymphomatosis, Cataracts in Chickens with, 647.
Lysogenicity in Staphylococcus Aureus of Animal Origin. Staphylococcic Phages. I., 832.

M

Macaca Mulatta Monkey, Hematological Effects of Acute and Chronic Experimental Blood Loss in the, 1081.
Magnetic Stirrer, New (Research Tool), 224.
Mammals, Comparison of Bacteriological and Serological Techniques for Detection of Leptospirosis in Wild, 192.
Mastitis, Experimental Viral Bovine, 819.
Mastitis, Parenteral Benzathine Penicillin V in Bovine, 287.
Mastitis-Producing Staphylococcus, a Coliform Organism, and Streptococcus Agalactiae, Activity in Milk of a, 736.
Mesenteric Abscesses in the Horse, Radiographic Diagnosis of, 448.
Mice Caused by Psorergates-Simplex Successfully Treated with Aramite, Follicular Acariasis of, 198.
Mice Vaccinated Against Swine Erysipelas with Adsorbed Bacterin, Interference of Anti-Hog Cholera Serum with the Immunity in, 558.
Microscope, Direct-Focusing Ultraviolet (Research Tool), 928.
Milk of a Mastitis-Producing Staphylococcus, a Coliform Organism, and Streptococcus Agalactiae, Activity in, 736.
Mink and Ferrets, Experimental Distemper in. II. Appearance and Significance of Histopathological Changes, 378.
Mink, Field Evaluation of Clostridium Botulinum Type C Toxoids in, 166.
Mink, The Digestibility of Animal Fats and Proteins by, 1086.
Mink with Systemic Proliferation of the Plasma Cells, Studies on a Disease in, 384.
Monkey, Hematological Effects of Acute and Chronic Experimental Blood Loss in the Macaca Mulatta, 1081.
Mouse, Colonic Intussusception in the, 750.
Mucoprotein of Siliceous Urinary Calculi of Bovine Origin, A Partial Chemical Analysis of the, 249.

XUM

Muscular Dystrophy in Lambs and Calves, A Succinoxidase Inhibitor in Feeds Associated with, 235.
Muscular Dystrophy in Lambs, The Relationship of Serum Glutamic Oxalacetic Transaminase to Nutritional, 75.
Mycoplasma Hyorhinis, and Nasal Trichomonads on Swine Tissue Cultures, Action of Certain Viruses, 1010.
Myoclonia Congenita, Studies on. III. Drugs and Other Factors Affecting Severity of Tremor in Pigs, 319.
Myoclonia Congenita, Studies on. IV. The Segmental Reflex in Normal and Affected Pigs, 627.
(Myopathy) in Lambs and Calves, White Muscle Disease. VI. Effects of Selenium and Vitamin E on Lambs, 231.

N

Nasal Discharge to Infection with Infectious Bovine Rhinotracheitis Virus, The Relationship of, 985.
(Nematoda) and Its Chicken Host, Anthelmintic Studies with Carbon 14-Labeled Carbon Tetrachloride on Ascaridia Galli, 662.
Nematodes, Studies on Experimental Concurrent Infections of Dairy Calves with Coccidia and. I. Eimeria Spp. and the Small Intestinal Worm, Cooperia Punctata, 281.
Nematodes, Studies on Experimental Concurrent Infections of Dairy Calves with Coccidia and. II. Eimeria Spp. and the Medium Stomach Worm, Ostertagia Ostertagi, 487.
Nematodes to the Action of Phenothiazine, Strain Variation on the Response of Sheep. IV. Efficacy of Single Therapeutic Doses for the Removal of Haemonchus Contortus, 670.
Nephelometry and a Nephelo-Culture Flask Used in Measuring Growth of Leptospires, 1072.
Nephritis, Effect of Nitrofurantoin on Experimental Canine Bacterial, 1020.
Nephroma in Swine, Embryonal, 324.
Newcastle Disease Infection, Factors Affecting Interference with, 352.
Newcastle Disease Vaccines, Role of Adjuvants in Immunogenicity of Killed, 357.
Newcastle Disease Virus Contaminants in Newcastle Disease Wing-Web Vaccines, Studies on Pathogenic, 946.
Newcastle Disease Virus in Chorioallantoic Membranes Suspended in Tyrode's Solution, Influence of Temperature on the Growth of, 111.
Newcastle Disease Virus in Aqueous Humor of Chickens, 545.
Newcastle Disease Virus in Tissue Culture, Titration of, 116.
Nitrofurantoin on Experimental Canine Bacterial Nephritis, Effect of, 1020.
3-Nitro-4-Hydroxyphenylarsonic Acid in Chicks and Poults, The Effect of Para-Aminobenzoic Acid on the Toxicity of, 655.

O

Ornithosis Caused by Agents of Low Virulence, Studies of Two Epornitics of, 935.
Ornithosis Epornitic in California Turkeys, Investigation of a Recent, 941.
Ornithosis in Herons and Egrets, Experimental, 884.
Ornithosis in Turkeys, Preliminary Studies on the Histopathology of Experimental, 341.
Ostertagia Ostertagi, Eimeria Spp. and the Medium Stomach Worm. Studies on Experimental Concurrent Infections of Dairy Calves with Coccidia and Nematodes. II., 487.
Ovary of the Domesticated Pig, Age-Change Studies of the, 578.
Ovine Calculosis, Preliminary Observations in Experimental. Ruminant Urolithiasis. I., 863.
Oxytetracycline on Chicken Spermatozoa, Studies of the Effect of, 957.

P

Paper Electrophoresis, Studies of Goat Serum Proteins by, 921.
Para-Aminobenzoic Acid on the Toxicity of 3-Nitro-4-Hydroxyphenylarsonic Acid in Chicks and Poults, The Effect of, 655.
Parasites, Field Tests on the Efficacy of Piperazine-Carbon Disulfide Complex in the Treatment of Foals for Gastrointestinal, 452.
Parasites of Cattle in Texas, Incidence of Certain Gastrointestinal, 766.
Parasites of the Horse, Critical Tests with Polymethylenepiperazine (PMP) Against, 69.
Parasites, Studies on Bovine Gastrointestinal. XX. The Results of Feeding Small Amounts of Phenothiazine in Pure Infections of Cooperia Punctata, 492.
Parasites, Studies on Bovine Gastrointestinal. XXII. The Rate of Consumption of Phenothiazine in Salt-Phenothiazine Mixtures by Cattle, 498.
Parasitism in Cattle, The Relationship of Diet to Gastrointestinal Helminth, 806.
Pasteurella Multocida, Studies on. IV. Serological Types from Species Other than Cattle and Swine, 173.
Pasteurellosis in Lambs, Septicemic, 94.
Pathology of Domestic Animals—A Study of 487 Cases, The Oral and Pharyngeal, 1032.
Penile Development of the Angora Goat, Observations on the, 603.
(Pharynx) The Oral and Pharyngeal Pathology of Domestic Animals—A Study of 487 Cases, 1032.
Phenolsulfonphthalein Clearance as a Measure of Renal Function in Ewes, 858.
Phenothiazine, A Comparison of the Anthelmintic Efficiency of Purified and N. F., 409.
Phenothiazine, Further Studies on the Relationship Between Particle Size and Anthelmintic Efficiency of, 201.
Phenothiazine in Pure Infections of Cooperia Punctata, The Results of Feeding Small Amounts of. Studies on Bovine Gastrointestinal Parasites. XX., 492.
Phenothiazine in Salt-Phenothiazine Mixtures by Cattle, Rate of Consumption of. Studies on Bovine Gastrointestinal Parasites. XXII., 498.
Phenothiazine, Strain Variation on the Response of Sheep Nematodes to the Action of. IV. Efficacy of Single Therapeutic Doses for the Removal of Haemonchus Contortus, 670.
Phosphate as an Anthelmintic in Cattle, Trial with a New Organic, 278.
Phosphate as an Anthelmintic in Sheep, Trial with a New Organic, 442.
Phosphates and a Carbamide as Anthelmintics in Ruminants, The Efficacy and Toxicity of Certain Organic, 784.
Phospholipase to the Hemolysin of Leptospira Pomona, Studies on the Relationship of a, 592.
Pig, Age-Change Studies of the Ovary of the Domesticated, 578.
Pig, Anatomic and Functional Studies of the Coronary Circulation in the Dog and, 18.
Pig Serum, A Pooled Human Serum as a Standard and the Effects of Freezing and Thawing on the Electrophoretic Pattern of Baby, 180.
Pig, The Changing Morphology in the Uterus of the Growing, 573.
Pigs, Drugs and Other Factors Affecting Severity of Tremor in. Studies on Myoclonia Congenita. III., 319.
Pigs, The Segmental Reflex in Normal and Affected. Studies on Myoclonia Congenita. IV., 627.
Pigs with Ascaris Suum, Experimental Infection of, 7.
Pigs with Transmissible Gastroenteritis Virus, The Effects of Infecting Newborn, 585.
Piperazine-Carbon Disulfide Complex in the Treatment of Foals for Gastrointestinal Parasites, Field Tests on the Efficacy of, 452.
Pipette, Automatic (Research Tool), 415.
Plaque Technique for the Study of Porcine Enteroviruses, The Use of the, 568.
Plasma Cells, Studies on a Disease in Mink with Systemic Proliferation of the, 384.
Plasma Glucose in Sheep Given Butyrate, Acidosis and, 430.
Plasma Sodium and Potassium in the Cow, The Effects of Four Synthetic Corticosteroids on Leukocytes, Blood Glucose and, 697.
Pneumatic Control System (Research Tool), 767.
Pneumonia, Characteristics of a Virus Associated with Lamb, 84.
Pneumonia in Lambs, Experimental Production of, 78.
Pneumonia in Slaughtered Lambs, Microbial Agents Associated with, 87.
Poisoning in Cattle, Sodium Metabisulfite and Sulfur Dioxide Gas (Silage Preservative), 690.
Polymethylenepiperazine (PMP) Against Parasites of the Horse, Critical Tests with, 69.
Porcine Enteroviruses, The Use of the Plaque Technique for the Study of, 568.
Potassium in the Cow, The Effects of Four Synthetic Corticosteroids on Leukocytes, Blood Glucose and Plasma Sodium and, 697.
Poults, The Effect of Para-Aminobenzoic Acid on the Toxicity of 3-Nitro-4-Hydroxyphenylarsonic Acid in Chicks and, 655.
Propiolactone, The Inactivation of Foot-and-Mouth Disease Virus, Type A-119, with Beta-, 992.
Proteins by Paper Electrophoresis, Studies of Goat Serum, 921.
Proteins by Mink, The Digestivity of Animal Fats and, 1086.
Psoroptes-Simplex Successfully Treated with Aramite, Follicular Acariasis of Mice Caused by, 198.

XUM

Pullorum Disease, Flocculation Tests for. II. A Study of Frozen Antigen Extract for Conducting the Microscopic Slide Test (Scientific Note), 1090.
Pulmonary Alveolar Wall, Electron Microscopy of the, 723.
Pyometra in Dogs, with Reference to the Hypothalamic-Hypophysial Neurosecretory System, Observations on, 41.

R

Rabbit Lung Cells, Multiplication and Cytopathogenic Effect of Foot-and-Mouth Disease Virus in Cultures of Fetal, 815.
Rabbit, Study of the Sperm Capacitation Factor in the Genital Tract of the Female, 753.
Rabbit Uteri, Investigations Concerning Bactericidal Substances in, 206.
Rabbits Infected with Eimeria Stiedae, Ionographic Studies of, 589.
Rabbits, The Effect of Antibiotics on Semen Production in, 756.
Rabies Virus, Attempts to Immunize Guinea Pigs with Flury Vaccine and Subsequent Challenge with Fixed, 145.
Radioactive Standards and Sources Set (Research Tool), 416.
Radioactivity Counter, Versatile (Research Tool), 224.
(Radioiodine) I^{131} Accumulation in the Thyroid Glands of Swine, Use of Scintillation Detector for Determining, 14.
Red Blood Cells After Parenteral Injection at Various Sites, The Absorption of, 1062.
Red Blood Cells, The Effects of Certain Diseases and Transfusion Methods on the Life Span of, 1096.
Reflex Control of the Ruminant Stomach with Special Reference to the Eructation Reflex, Studies of the, 461.
Research Tools:
  Automatic Pipette, 415.
  Bacteriological Incubator, 608.
  Coordinated Plate and Film Comparator, 224.
  Cylinder-Type Filter, 1093.
  Demand-Breathing Respirator, 928.
  Direct-Focusing Ultraviolet Microscope, 928.
  Disposable Plastic Caps and Hoods, 1093.
  Escape-Proof Container, 414.
  Flame Photometer, 607.
  Fraction Collecting Device, 608.
  Hemoglobinometer, 928.
  Medical Crematory Destructor, 223.
  Microhematocrit Adapter, 1091.
  Miniature pH Electrode Assembly, 416.
  Negative Viewer, 768.
  New Distillation Unit, 415.
  New Magnetic Stirrer, 224.
  New Stereomicroscope, 1091.
  Pneumatic Control System, 767.
  Portable Transistor Cardiac Pacemaker, 1092.
  Prepared Plastic Plates, 1092.
  Quarter Milker, 1092.
  Radioactive Standards and Sources Set, 416.
  Recording Camera, 768.
  Transistor Electrocardiogram Marker, 927.
  Versatile Radioactivity Counter, 224.
Respirator, Demand-Breathing (Research Tool), 928.
Respiratory Disease, Isolation and Characterization of Cytopathogenic Enteroviruses from Cattle with, 27.
Reticuloperitonitis, The Hematology of Chronic Bovine, 255.
Rhinotracheitis—The Occurrence of Intranuclear Inclusion Bodies in Experimentally Infected Animals, Infectious Bovine, 505.
Rhinotracheitis Virus, The Relationship of Nasal Discharge to Infection with Infectious Bovine, 985.
Ruminant Forestomach, The Presence of Lipids in the Epithelium of the, 437.
  Correction: 783.
Ruminant Stomach with Special Reference to the Eructation Reflex, Studies of the Reflex Control of the, 461.
Ruminants and Their Simultaneous Determination, The Body Fluids of, 184.
Ruminants, The Efficacy and Toxicity of Certain Organic Phosphates and a Carbamide as Anthelmintics in, 784.

S

Salmonella Gallinarum Infection in Turkey Poults with Chloramphenicol and Furazolidone, The Treatment of, 659.
Salmonella Pullorum, Susceptibility of Certain Strains of White Leghorn Chickens to, 350.
SCIENTIFIC NOTES—See also RESEARCH TOOLS
Scientific Notes:
  Animal Care Panel Meeting, 927.
  Conference on Histopathology of Experimental Avian Lymphomatosis, 223.
  Flocculation Tests for Pullorum Disease. II. A Study of Frozen Antigen Extract for Conducting the Microscopic Slide Test, 1090.
  Foundation Announces Establishment of Research Advisory Service on Information Processing, 414.
  Incidence of Certain Gastrointestinal Parasites of Cattle in Texas, 766.
  Instrument Symposium and Research Equipment Exhibit, 927.
  Microcard Reprints of Research Journal, 607.
  Report Made on American Scientists' Knowledge of Foreign Languages, 414.
  Serological Classification of Escherichia Coli, 1090.
  Southern Conference on Avian Diseases, 927.
  Symposium on Antibiotics, 767, 927.
Scintillation Detector for Determining I^{131} Accumulation in the Thyroid Glands of Swine, Use of, 14.
Selenium and Vitamin E on Lambs, Effects of. White Muscle Disease (Myopathy) in Lambs and Calves. VI., 231.
Semen Production in Rabbits, The Effect of Antibiotics on, 756.
Serum, A Pooled Human Serum as a Standard and the Effects of Freezing and Thawing on the Electrophoretic Pattern of Baby Pig, 180.
Serum Cholesterol and Paper Electrophoretic Lipoprotein Pattern in Cattle, 989.
Serum, Electrophoretic Studies of Bovine. I. Changes in the Serum Proteins After Splenectomy, 270.
Serum Glutamic Oxalacetic Transaminase to Nutritional Muscular Dystrophy in Lambs, The Relationship of, 75.
Serum Protein Patterns of Sheep as Determined by Paper Electrophoresis, The Effects of Freezing and Storage at —20 C. on, 434.
Serum Proteins by Paper Electrophoresis, Studies of Goat, 921.
Serum with the Immunity in Mice Vaccinated Against Swine Erysipelas with Adsorbed Bacterin, Interference of Anti-Hog Cholera Hyperimmune, 558.
Sheep and Goats, Experimental Strongyloidiasis in. I. Single Infections, 102.
Sheep, and Springbok Antelope, Using Glycine-2-C^{14}, Erythrocyte Survival Studies in the Mule Deer, Aoudad, 917.
Sheep as Determined by Paper Electrophoresis, The Effects of Freezing and Storage at —20 C. on Serum Protein Patterns. of, 434.
Sheep Erythrocytes by Specific Antiserums, Agglutination of Polysaccharide-Sensitized. Characterization of Vibrio Fetus Antigens. II., 154.
Sheep Following Oral Administration of Vibrio Fetus, Bacteremia in Pregnant, 677.
Sheep from Northeastern and Northern Brazil, Copper and Iron in Livers of Undernourished Cattle and, 247.
Sheep Given Butyrate, Acidosis and Plasma Glucose in, 430.
Sheep Nematodes to the Action of Phenothiazine, Strain Variation on the Response of. IV. Efficacy of Single Therapeutic Doses for the Removal of Haemonchus Contortus, 670.
Sheep to Listeria Monocytogenes and Pilot Studies on Vaccination, Acquired Resistance of, 966.
Sheep, Trial with a New Organic Phosphate as an Anthelmintic in, 442.
Sheep Using a Single Dose of X-Irradiated Larvae, Studies on Immunity to Haemonchus Contortus Infection —Vaccination of, 527.
(Silage Preservative) Poisoning in Cattle, Sodium Metabisulfite and Sulfur Dioxide Gas, 690.
Skin Grafting, Canine Autogenous, 898.
Skin of Hereford and Aberdeen Angus Cattle, The Comparative Histology of the, 61.
Sodium Chloride, The Control by Force Feeding. Studies on Urolithiasis. III., 423.
Sodium Metabisulfite and Sulfur Dioxide Gas (Silage Preservative) Poisoning in Cattle, 690.
Sperm Capacitation in the Genital Tract of the Female Rabbit, Study of the, 753.
Spermatozoa, Studies of the Effect of Oxytetracycline on Chicken, 957.
Splenectomy, Changes in the Serum Proteins After. Electrophoretic Studies of Bovine Serum. I., 270.
Staphylococci Associated with the Bovine Udder, A Study of the, 825.
Staphylococcic Phages. I. Lysogenicity in Staphylococcus Aureus of Animal Origin, 832.
Staphylococcic Phages. II. The Use of Human Typing Phages and Adapted Human Typing Phages in the Typing of Staphylococcus Aureus of Animal Origin, 835.
Staphylococcic Phages. III. Typing of Staphylococcus Aureus Cultures Isolated from the Bovine Udder, 838.

Staphylococcus, a Coliform Organism, and Streptococcus Agalactiae, Activity in Milk of a Mastitis-Producing, 736.
Staphylococcus Aureus of Animal Origin, Lysogenicity in. Staphylococcic Phages. I., 832.
Staphylococcus Aureus of Animal Origin, The Use of Human Typing Phages and Adapted Human Typing Phages in the Typing of. Staphylococcic Phages. II., 835.
Staphylococcus Aureus Cultures Isolated from the Bovine Udder, Typing of. Staphylococcic Phages. III., 838.
Stenosis of the Pulmonary Artery in Calves, Experimental, 265.
(Sterilization) Sterilized Cultures and Filtrates, Udder Reaction to, 718.
Stomach Worm, Ostertagia Ostertagi, Eimeria Spp. and the Medium. Studies on Experimental Concurrent Infections of Dairy Calves with Coccidia and Nematodes. II., 487.
Streptococcus Agalactiae, Activity in Milk of a Mastitis-Producing Staphylococcus, a Coliform Organism, and, 736.
Strongyle Larvae in Vitro, Screening Tests of Carbamates and Related Compounds Against Horse, 300.
Strongyloidiasis in Sheep and Goats, Experimental. I. Single Infections, 102.
Succinoxidase Inhibitor in Feeds Associated with Muscular Dystrophy in Lambs and Calves, A, 235.
Sulfabromomethazine, Treatment of Experimental Bovine Coccidiosis with Multiple Small Doses and Single Large Doses of Sulfamethazine and, 708.
Sulfamethazine and Sulfabromomethazine, Treatment of Experimental Bovine Coccidiosis with Multiple Small Doses and Single Large Doses of, 708.
Sulfapyrimidines—Bromosulfamerazine and Bromosulfamethazine, The Pharmacological Action Developed in Cattle by Two Halogenated, 483.
Sulfobromophthalein Sodium Retention Tests as an Index to Canine Liver Dysfunction. A Comparison of Uric Acid Determinations and, 372.
Sulfonamide Excretion in the Cow, Rates and Routes of. I. Milk Levels Following Single Intravenous and Oral Administrations, 714.
Sulfur Dioxide Gas (Silage Preservative) Poisoning in Cattle, Sodium Metabisulfite and, 690.
Swine, Embryonal Nephroma in, 324.
Swine Erysipelas Bacterins and Vaccines in Weanling Pigs, with Particular Reference to the Status of Their Dams, Comparative Experimental Evaluation of, 1002.
Swine Erysipelas with Adsorbed Bacterin, Interference of Anti-Hog Cholera Hyperimmune Serum with the Immunity in Mice Vaccinated Against, 558.
Swine, Hematological Studies of Normal, Ascarid-Infected, and Hog Cholera-Vaccinated, 562.
Swine, Influence of Oral Intake of Vitamin B_{12} on Total Blood Serum Vitamin B_{12} Levels in, 338.
Swine Kidney Cell Cultures—Susceptibility to Viruses and Use in Isolation of Enteric Viruses of Swine, 127.
Swine, The Effect of Intramuscular Injections of Vitamin B_{12} on Blood Serum Levels in, 333.
Swine Tissue Cultures, Action of Certain Viruses, Mycoplasma Hyorhinis, and Nasal Trichomonads on, 1010.
Swine, Use of Scintillation Detector for Determining I^{131} Accumulation in the Thyroid Glands of, 14.
Swine with Brucella Melitensis (Mediterranean Type), Experimental Infection of, 634.
Swine with Brucella Neotomae, Experimental Infection of, 872.
Swine with Enteritis, Isolation of a Cytopathogenic Agent from, 625.

T

Thyroid Glands of Swine, Use of Scintillation Detector for Determining I^{131} Accumulation in the, 14.
Tissue Culture of Avian Visceral Lymphoid Tumors and in Vitro Serial Passage of the Virus, 119.
Tissue Culture, Titration of Newcastle Disease Virus in, 116.
Tissue Cultures, Action of Certain Viruses, Mycoplasma Hyorhinis, and Nasal Trichomonads on Swine, 1010.
Tremor in Pigs, Drugs and Other Factors Affecting Severity of. Studies on Myoclonia Congenita. III., 319.
Trichomonads on Swine Tissue Cultures, Action of Certain Viruses, Mycoplasma Hyorhinis, and Nasal, 1010.
Trichostrongylus Axei (Cobbold, 1879), Studies on. IV. Some Aspects of Treatment, Pathogenicity, and Quantification in Experimental Infections of a Horse Strain in Calves, 787.
Trypsin-Modified Infectious Bronchitis Virus, Hemagglutination by, 876.
Tumors and in Vitro Serial Passage of the Virus, Tissue Culture of Avian Visceral Lymphoid, 119.
Turkey Poults with Chloramphenicol and Furazolidone, The Treatment of Salmonella Gallinarum Infection in, 659.
Turkeys, Investigation of a Recent Ornithosis Epornitic in California, 941.
Turkeys, Preliminary Studies on the Histopathology of Experimental Ornithosis in, 341.
Turkeys, Spontaneous Cataracts in, 961.

U

Udder, A Study of the Staphylococci Associated with the Bovine, 825.
Udder Reaction to Sterilized Cultures and Filtrates, 718.
Udder, Typing of Staphylococcus Aureus Cultures Isolated from the Bovine. Staphylococcic Phages. III., 838.
Urethral Epithelium of Domestic Animals, Histology of the, 941.
Uric Acid Determinations and Sulfobromophthalein Sodium Retention Tests as an Index to Canine Liver Dysfunction, A Comparison of, 372.
Urinary Calculi of Bovine Origin, A Partial Chemical Analysis of the Mucoprotein of Siliceous, 249.
Urine, Identification and Determination of Kynurenin and 3-Hydroxykynurenin in Bovine, 397.
Urolithiasis, Ruminant. I. Preliminary Observations in Experimental Ovine Calculosis, 863.
Urolithiasis, Studies on. III. The Control by Force Feeding Sodium Chloride, 423.
Urolithiasis, Studies on. IV. The Effects of the Ration on the Predisposition as Measured by the Urinary Muceproteins, 426.
Uteri, Investigations Concerning Bactericidal Substances in Rabbit, 206.
Uterus of the Growing Pig, The Changing Morphology in the, 573.

V

Vaccination, Acquired Resistance of Sheep to Listeria Monocytogenes and Pilot Studies on, 966.
Vaccination of Sheep Using a Single Dose of X-Irradiated Larvae, Studies on Immunity to Haemonchus Contortus Infection—, 527.
Vaccination with Irradiated Larvae, Immunological Studies on Dictyocaulus Viviparus Infection in Calves—Double, 522.
Vaccine and Subsequent Challenge with Fixed Rabies Virus, Attempts to Immunize Guinea Pigs with Flury, 145.
Vaccines in Weanling Pigs, with Particular Reference to the Status of Their Dams, Comparative Experimental Evaluation of Swine Erysipelas Bacterins and, 1002.
Vaccines, Role of Adjuvants in Immunogenicity of Killed Newcastle Disease, 357.
Vaccines, Studies on Pathogenic Newcastle Disease Virus Contaminants in Newcastle Disease Wing-Web, 946.
Vibrio, Biochemical and Serological Separation of Some Members of the Genus, 532.
Vibrio Fetus Antigens, Characterization of. I. Chemical Properties and Serological Activities of a Soluble Antigen, 148.
Vibrio Fetus Antigens, Characterization of. II. Agglutination of Polysaccharide-Sensitized Sheep Erythrocytes by Specific Antiserums, 154.
Vibrio Fetus, Bacteremia in Pregnant Sheep Following Oral Administration of, 677.
Vibriosis of Ewes, Antibiotics for the Control of, 973.
Viewer, Negative (Research Tool), 768.
Virus in Tissue Culture, Isolation and Propagation of Bluetongue, 398.
Virus, In Vitro Propagation of Hog Cholera. II. Some Biological and Immunological Characteristics of Hog Cholera Virus Grown in Tissue Culture, 304.
Virus, In Vitro Propagation of Hog Cholera. III. Cultivation of an Immunological Variant, with Retention of Its Identifying Characteristics, 311.
Viruses, Mycoplasma Hyorhinis, and Nasal Trichomonads on Swine Tissue Cultures, Action of Certain, 1010.
Vitamin B_{12} Deficiency in Cattle by Cobalt Pellet Therapy, The Correction of Cobalt or, 977.
Vitamin B_{12} on Blood Serum Levels in Swine, The Effect of Intramuscular Injections of, 333.
Vitamin B_{12} on Total Blood Serum Vitamin B_{12} Levels in Swine, Influence of Oral Intake of, 338.
Vitamin E on Lambs, Effects of Selenium and. White Muscle Disease (Myopathy) in Lambs and Calves. VI., 231.

W

Western Equine Encephalomyelitis Virus in Tissue Culture and Color Test Titration of Virus and Its Antibodies in Ehrlich Cells, 459.

XUM

White Leghorn Chickens to Salmonella Pullorum, Susceptibility of Certain Strains of, 350.
White Muscle Disease (Myopathy) in Lambs and Calves. VI. Effects of Selenium and Vitamin E on Lambs, 231.
Worm, Cooperia Punctata, Eimeria Spp. and the Small Intestinal. Studies on Experimental Concurrent Infections of Dairy Calves with Coccidia and Nematodes. I., 281.

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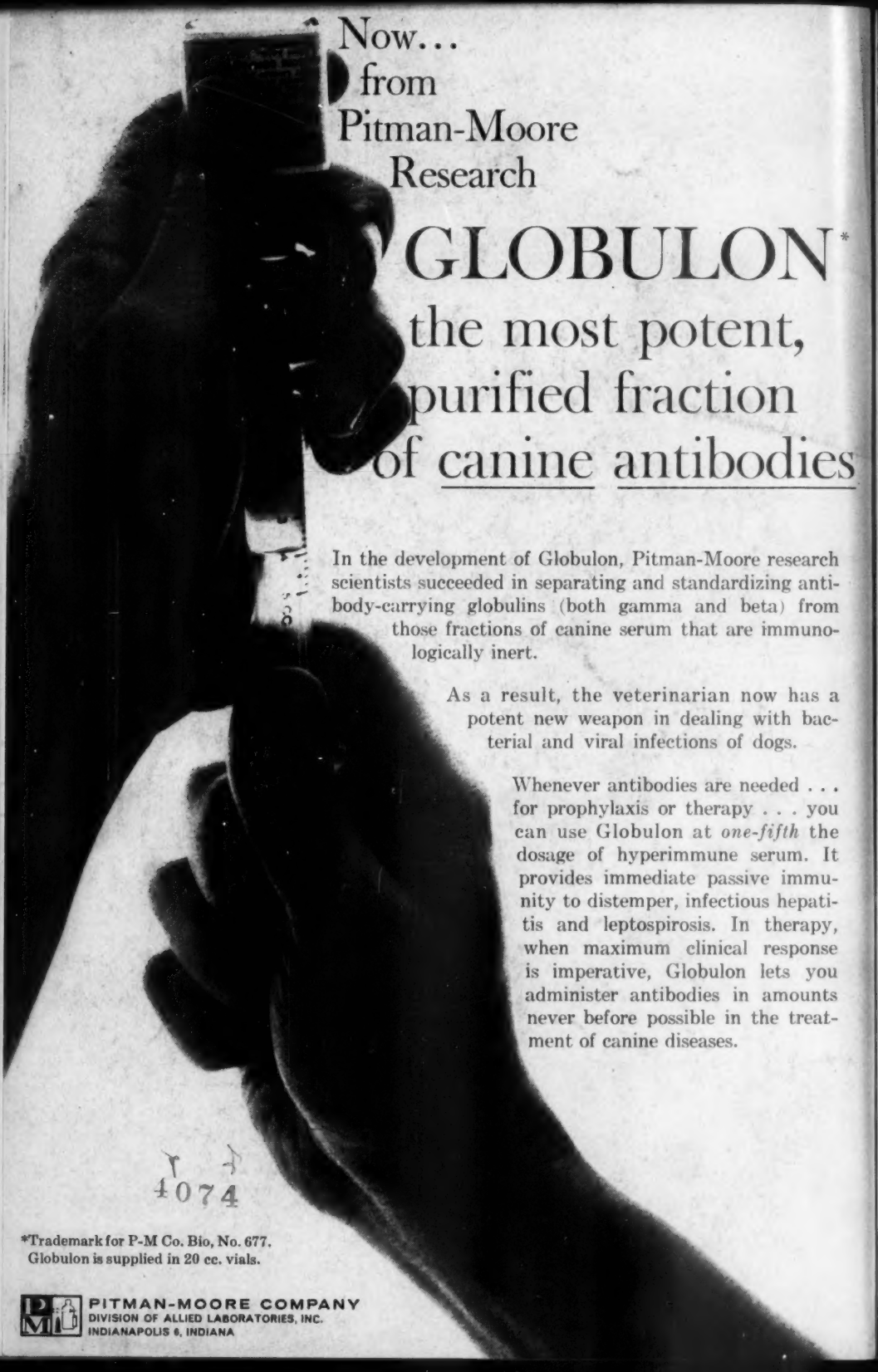

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GLOBULON*

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In the development of Globulon, Pitman-Moore research scientists succeeded in separating and standardizing antibody-carrying globulins (both gamma and beta) from those fractions of canine serum that are immunologically inert.

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Globulon is supplied in 20 cc. vials.

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